Universidade de Aveiro Departamento de Química
2015

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Complexos de Flavonóides e Ciclodextrinas e sua incorporação em alimentos

Flavonoid-Cyclodextrin complexes and their incorporation in milk products

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo de Bioquímica Alimentar, realizada sob a orientação científica da Doutora Susana Isabel Fonseca de Almeida Santos Braga, Investigadora Principal do Departamento de Química da Universidade de Aveiro, da Doutora Maria Paula Matos Marques Catarro, Professora Auxiliar do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologias da Universidade de Coimbra, e da Doutora Aida Maria Gonçalves Moreira da Silva, Professora Adjunta da Escola Superior Agrária de Coimbra.

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palavras-chave

Conservantes alimentares, quercetina, encapsulação molecular, ciclodextrinas β e γ, FTIR, difracção de raios-x de pós, termogravimetria, ensaios de capacidade antioxidante, fortificação alimentar.

resumo

O trabalho apresentado descreve a inclusão do flavonóide quercetina nas ciclodextrinas β e γ, com posterior incorporação dos complexos em laticínios, nomeadamente queijo fresco. A caracterização dos complexos de inclusão foi feita utilizando várias técnicas, tendo sido também realizados ensaios antioxidantes para avaliar a sua capacidade antioxidante e de anti-peroxidação lipídica, em comparação com a quercetina. A incorporação dos complexos no queijo fresco resultou na modificação de algumas características do produto alimentar, tendo estes complexos também apresentado uma promissora capacidade antioxidante.

keywords

Food preservatives, quercetin, molecular encapsulation, cyclodextrins β and γ , FTIR, powder XRD, thermogravimmetry, antioxidant capacity assays, food fortification.

abstract

The presented work describes the inclusion of the flavonoid quercetin into β and γ cyclodextrins and the subsequent incorporation of such complexes into a dairy product — fresh cheese. The characterization of the complexes was made using various techniques, and antioxidant assays were also performed to assess their antioxidant and anti-lipid peroxidation capacity in comparison to quercetin. The incorporation of the complexes in fresh cheese resulted in the modification of some of the characteristics of the food product, having these also presented promising antioxidant capacity.

Contents

1. Introduction

1. Introduction

One of the main concerns of the food industry is the maintenance of the stability and quality of food items. Due to its complex nature, food is susceptible to external factors which can hinder and damage its structure and characteristics in a process named food deterioration. The main causes are microorganisms (such as bacteria, yeast and mould), enzymes, chemical changes due to water content, heat, metals, oxygen from air or light, contamination by soils, stones and insects, and physical damage (1). In order to avoid or prevent food spoilage, several methods have been developed. These may be physical processes, such as heating or cooling, lowering of the water content through drying or dehydration, filtration, sterilization, irradiation, high pressuring and vacuum packaging; chemical, like salting, smoking, addition of sugar or artificial acidification; or biochemical, through lactic or alcoholic fermentation (2).

A modern and increasingly used method for food preservation is the addition of synthetic substances to help maintain its characteristics and to improve its preservation (and thus, shelf life) and quality. These comprise antioxidants, emulsifiers, stabilizers, acidity regulators, preservatives and encapsulating agents (3). Noteworthy, interests and research in antioxidants are growing, making it one of the most important food additive groups of the present day. The main function of antioxidants is to control or slow down the oxidation processes that occur in food with a usually undesirable outcome: rancidity, spoilage and off-flavours. Antioxidants may be of natural sources, such as phenolic compounds, or synthetic, such as butylated hydroxytoluene (BHT) (4).

Antioxidants bring many advantages to food preservation. Nonetheless, a great challenge for food processing techniques is their own preservation inside the food products, in order to retain their activity. The unstable nature and high reactivity of antioxidants means that they are easily degraded, rapidly losing antioxidant capacity (5). One technique that has the ability to protect food additives is microencapsulation, a process in which tiny particles or droplets are surrounded by a coating, or embedded in an homogenous or heterogeneous matrix, to give small capsules with many useful properties (6). Various microencapsulation techniques are currently available, and the microencapsulated products are widely used in

food products, pharmaceutical and cosmetic industries, personal care, agricultural products, veterinary medicine, industrial chemicals, biotechnology, biomedical and sensor industries (5).

1.1 Mechanisms of Food Deterioration

Food deterioration, as mentioned before, is caused by a wide variety of factors. For the purpose of this work, this chapter will focus only on the two food deterioration processes which are the most relevant for the field of biochemistry related with food deterioration: microbiological deterioration and oxidation. The former will cover the mechanisms through which food is spoiled by microorganisms, namely bacteria, mould and yeast, and oxidation will expose the mechanisms of protein and lipid peroxidation, explaining how they occur and what are the consequences for the quality of food products that undergo such processes.

1.1.1 Microbiological Deterioration of Food

Food deterioration in a very advanced state, with deep alteration in its characteristics (physical, chemical and organoleptic) which renders it unacceptable for consumption, is called food spoilage. These changes turn the spoiled foods into dangerous products, with consequences for the health of the consumers (7). Microbial spoilage of foods arises from the growth and metabolism of agents like bacteria, mould and yeast, and is one of the most common causes of spoilage, manifesting itself as visible microbiological growth, textural changes or as off-odours and off-flavours (8).

Bacterial spoilage of food is caused mainly by lactic acid bacteria (LAB), *Pseudomonas* and *Enterobacteriaceae*. Lactic acid bacteria spoils food through the production of organic acids such as lactic and acetic acids, ethanol and CO_2 from homofermentative and heterofermentative metabolic pathways, causing the development of off-flavours and slime (9). *Pseudomonas spp*. may cause spoilage of dairy products through the production of extracellular enzymes (like protease and lecitinase) that are

heat-stable and can therefore remain active during pasteurization or ultra-high-temperature treatments (10). *Enterobacteriaceae* (enterobacteria) have a role in food spoilage due to their ability to metabolize amino acids to malodorous volatile molecules, such as foul-smelling diamines and sulfuric compounds (11).

Spoilage associated with mould is also very common, as these agents are able to grow on all kinds of food to cause not only organoleptic changes as off-flavours, discolouration and rotting, but also affording hazardous toxins and forming pathogenic or allergenic propagules (spores). The deterioration of food sensorial properties is often due to the production of enzymes during the growth of the mold, namely lipases, proteases and carbohydrases (12). Moulds may also produce volatile compounds such as dimethyldisulphide, geosmin and 2-methylisoborneol, which can affect the quality of foods and beverages even when present in very small amounts (13). The most important aspect of mould spoilage of foods is, however, the formation of mycotoxins. Over 400 mycotoxins are known today, aflatoxins being the best known, and the number is increasing rapidly. Mycotoxins are secondary metabolites which are toxic to vertebrate animals in small amounts. The toxicity of these metabolites is very different, with the so-called chronic toxicosis being the most important to humans (14).

Yeasts are unicellular fungi capable of converting carbohydrates to carbon dioxide and alcohols through fermentation of sugars such as glucose and sucrose. The most commonly used yeast in the food industry is the species *Saccharomyces cerevisiae*, present in most baking processes and alcoholic fermentation of beverages (15). Food spoilage of foods due to yeast occurs in the wine industry, for example, before fermentation or in its early stages, with the production of undesirable metabolic end compounds like ethyl acetate and acetate, which will affect the sensorial characteristics of the product (16).

Current research in the field of microbiological threats to food quality is directed towards the detection and characterization of the various strands of microorganisms which arise in food products. M.I. Luque *et al* developed a PCR method to detect ochratoxigenic mould strains (regardless of genus and species) in food products that can be used routinely in the HACCP (Hazard Analysis and Critical Control Points) programs of the food industry (17) and A.C.d.S. Pires *et al* tested the application of polydiacetylene (PDA) vesicles for the

detection of bacteria in food systems, verifying that surfactant-functionalized PDA vesicles undergo a colorimetric transition induced by molecules released by *S. aureus* and *E. coli*, a result that shows potential for this technique to be used in bacterial detection tests as a colorimetric biosensor (18). Regarding yeasts, due to their vast application in the wine industry, there are many studies currently directed for this field. A recent study by Y.P. Maturano *et al* analysed the dynamics of yeast population during pre-fermentative cold soak of Cabernet Sauvignon and Malbec wines, to find that the temperature at which it was conducted affected the natural proportion of the yeast species. When carrying the pre-fermentative cold soak at a temperature of 14°C, there was a significant increase in the total yeast counts. Furthermore, the high proportion of native *S. cerevisiae* in musts after the cold soak treatment affected the dominance of the added commercial starter, and the alcoholic fermentation of the wines was rather carried out by a native *S. cerevisiae* strain, with consequences to the sensory perception of wines (19).

1.1.2 Oxidation as a Mechanism for Food Deterioration

Redox reactions are the basis for numerous biochemical pathways, cellular chemistry, biosynthesis and regulation. While reductant and oxidant are chemical terms, in biological environments, they should be termed antioxidant and pro-oxidant, respectively (20). In food, the main agents responsible for oxidation are reactive oxygen species (ROS), a collective term that includes oxygen radicals and nonradical derivatives of oxygen. The oxygen radicals are superoxide anion ($O_2^{\bullet -}$), hydroxy ($HO^{\bullet}$), peroxy ($ROO^{\bullet}$), alkoxy ($RO^{\bullet}$), and hydroperoxy ($HOO^{\bullet}$) radicals. Nonradical derivatives are hydrogen peroxide (H_2O_2), ozone (O_3), and singlet oxygen ($^1O_2$). Superoxide anion, hydrogen peroxide, and hydroxy radicals are formed by a sequential univalent reduction of molecular triplet oxygen, while singlet oxygen is commonly formed by the excitation of triplet oxygen in the presence of a sensitizer and light (21). ROS can be produced during the normal metabolism of a biological system, including mitochondrial electron transportation reactions, or peroxisomal fatty acid metabolism. ROS may also occur as products of enzyme catalysis by nitric oxide synthases, NADPH oxidase, xanthine oxidase, lipoxygenases,

glucose oxidase, cyclooxygenases and the cytochrome P450 superfamily. ROS can be generated by exposure to exogenous agents including ultraviolet (UV) light, irradiation (X-rays, γ-rays, and visible light in the presence of a sensitizer), chemical agents (metal ions such as Fe^{3+} and Cu^{2+} and H_2O_2) and environmental pollutants (22). These oxygen species react with lipids, proteins, sugars, and vitamins to destroy essential fatty acids, amino acids and vitamins, or to produce undesirable volatile compounds and, more importantly, to produce carcinogens which render food products less acceptable or even unsuitable for consumption (23).

1.1.3 Protein Oxidation

Oxidation of proteins is believed to proceed via a free radical chain reaction. The abstraction of a hydrogen atom by a ROS leads to the generation of a protein carbon-centered radical ($P^{\bullet}$) which is consecutively converted into a peroxyl radical ($POO^{\bullet}$) in the presence of oxygen, and an alkyl peroxide (POOH) by abstraction of a hydrogen atom from another molecule. Further reactions with $HO_2^{\bullet}$ lead to the generation of an alcoxyl radical ($PO^{\bullet}$) and its hydroxyl derivative (POH). The nature of the resulting protein oxidation products depends strongly on the amino acids involved and how the oxidation process is initiated. The side chains of arginine, lysine and proline are oxidized to carbonyl residues through metal-catalyzed reactions, while cysteine and methionine are involved in cross-linking or yield sulfur-containing derivatives (24). The oxidative changes in proteins thus include cleavage of peptide bonds, modification of amino acid side chains and formation of covalent intermolecular cross-linked protein derivatives (25) (Figure 1).

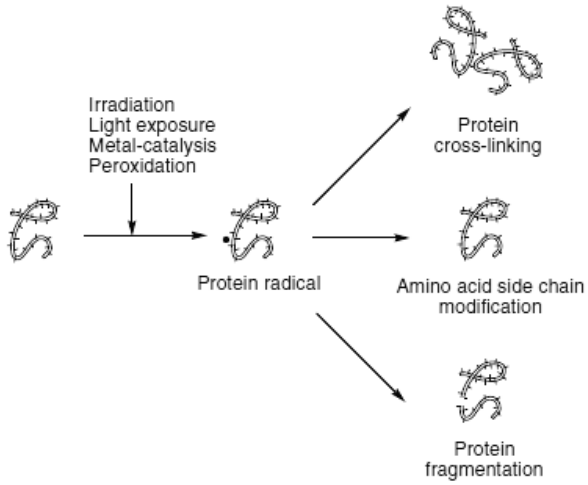


Fig. 1. Consequences of protein oxidation. From (25).

The high content of protein in food items of animal sources makes them the most susceptible to protein oxidation. In beef and chicken, the most affected proteins are myosin, troponin T, enolase and creatine kinase (26,27), which are the same for fish, with addition of actin (28). In milk, casein and β-lactoglobulin are the most oxidised proteins (29). Regarding the effects of protein oxidation on the quality of the food products, P.I. Zakrys-Waliwander *et al* showed that a high oxygen atmosphere packaging has negative effects in the tenderness of meat, due to the formation of oxidation-induced cross-links by myosin caused by oxidation (30). S. Traore *et al* investigated the effects of heat treatment on protein oxidation in pig meat, and verified that after being subjected to 100°C for 10 and 30 minutes, the meat samples showed increasing surface hydrophobicity, carbonyl products and protein aggregates, revealing oxidized myosin and actin. These oxidation products were significantly correlated with drip loss, suggesting a possible reduced ability of oxidized proteins to retain water (31).

1.1.4 Lipid Oxidation

Lipid oxidation in food systems is one of the most important factors affecting food quality, nutrition, safety, colour and consumers' acceptance. Controlling lipid oxidation remains an ongoing challenge as most foods comprise very complex matrices. Lipids are mostly incorporated as emulsions, and chemical reactions occur at various interfaces throughout the food matrix. Fats and oils in food can undergo oxidative deterioration through autoxidation, photosensitized oxidation, thermal oxidation or enzymatic oxidation (25). Autoxidation of lipids is a spontaneous reaction of molecular oxygen with lipids, leading to oxidative deterioration. It takes place by a free radical chain mechanism, similar to the one occurring in proteins, which involves three steps (Scheme 1): (1) the initiation step, where an homolytic hydrogen atom is abstracted from a methylene group, leading to the formation of an alkyl radical ($R^{\bullet}$); (2) the propagation step, involving the formation of peroxy radicals ($ROO^{\bullet}$) able to react with unsaturated fatty acids and form hydroperoxides (ROOH); and the termination step (3) when there is a formation of non-radical products by interaction of $R^{\bullet}$ and $ROO^{\bullet}$ (32).

(1) $RH \longrightarrow R^{\bullet}$

(2) $R^{\bullet} + O_2 \rightleftharpoons ROO^{\bullet}$

$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$

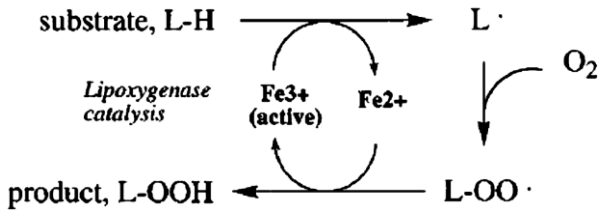
(3) $R^{\bullet} + R^{\bullet} \longrightarrow RR$

$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR$

$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2$

Scheme 1. Free radical chain reaction mechanism of autoxidation of lipids ($R^{\bullet}$ – fatty acid radical; ROOH – fatty acid hydroperoxide and $ROO^{\bullet}$ – peroxy radical). Adapted from (33).

Photosensitized oxidation occurs upon light excitation of photosensitizers, such as chlorophylls, affording mainly hydroperoxides and eventually also generating a few free radicals. In this process, the excited chlorophylls reacts with triplet oxygen to afford singlet oxygen species, which, in turn, will interact with unsaturated lipid substrates (such as polyunsaturated fatty acids, PUFA) producing allylic hydroperoxides (34). Thermal oxidation is induced by heating of the compounds, and follows the mechanism of autoxidation, with a faster reaction rate (35). Enzymatic oxidation of lipids is a result of the activity of lipoxygenase (LOX), which catalyzes the regio- and stereo-specific dehydrogenation of PUFAs containing a (1Z,4Z)-pentadiene system, like linoleic acid (LA), α-linolenic acid (α-LeA), or arachidonic acid (36)(Scheme 2).



Scheme 2. Role of lipoxygenase in the formation of lipid radicals. (L represents a lipid molecule. From (36).

The active enzyme abstracts an hydrogen atom from the methylene group of a polyunsaturated fatty acid, with the Fe(III) of its active site being reduced to Fe(II). Oxidation back to Fe (III) occurs by interaction with fatty acid hydroperoxides or hydrogen peroxide. A conjugated diene system is formed, followed by reaction with oxygen, yelding a peroxyl radical and ending in hydroperoxide generation (37).

Although hydroperoxides are nonvolatile and odourless, they are relatively unstable compounds, decomposing either spontaneously or in catalysed reactions to form volatile compounds of unpleasant odour (38). Examples of oxidative off-flavours are the beany flavours that commonly develop in soybean oil, the fishy flavours in fish oil, and creamy or metallic flavours that may develop in milk fat. Besides these rancid flavours, oxidative deterioration of lipids may cause the food bleaching due to the degradation of pigments, especially carotenoids, by the reactive intermediates. Free radicals may also lead to a reduction of nutritional quality by oxidising vitamin E (39). F.J. Hidalgo *et al* studied the formation of histamine from histidine in the presence of lipid hydroperoxides, and confirmed that amino acids can be decarboxylated in the presence of lipid oxidation products at moderate temperatures, suggesting the potential contribution of these reactions to biogenic polyamine formation in food systems (40). M.L. Ibargoitia *et al* used ^{1}H NMR to follow the evolution of margarines of varied compositions when heated at 180° C in an oven with aeration, paying special attention to the effect of enrichment with polyunsaturated acyl groups. Heating caused degradation of polyunsaturated acyl groups, depending not only on their unsaturation degree but also on the concentration of the different acyl groups, and hydrolysis reactions that lead to a reduction in 1-monoglycerides and an increase in 1,2-diglycerides (particularly in margarines with a higher water content) as well as degradation of some vegetable sterols (41).

1.2 Prevention and Inhibition of Food Deterioration

The food industry continues to develop efforts to ensure that food reaches the consumers in its best conditions. Currently there is a plethora of methods to prevent or inhibit food deterioration and spoilage, such as correct packaging, transportation conditions and incorporation of additives to preserve or enhance its characteristics and help maintain its quality (42). Traditional preservation techniques, such as smoking, salting, canning and freezing, are mainly based on physical methods, which manipulate factors like temperature and water content. The modern food additives, in turn, are substances that perform specific roles and functions, either increasing shelf life or inhibiting the growth of harmful microbes. They may also convey desired colours, odours or a specific flavour (3).

Food additives can have a natural origin, existing in a food either intrinsically or upon addition of natural substances; they may also be entirely artificial, implying that they are synthetically produced and not copies of any compounds found in nature (3).

1.2.1 Preservation of Food Through Additives

Because of the complex nature of food products, techniques that exert external water and energy transfers, like the traditional methods mentioned above, although effective, might be hard to optimize and control, demanding many check points and complex equipment. The elimination of possible microbial contamination through these physical methods is not always possible, which creates the need for innovative preservation methods able to guarantee the safety of the product (43). Preservatives are chemical substances capable of retarding or arresting microorganism growth. They can be used to prevent a variety of processes, namely fermentation, acidification, decomposition, oxidation and browning, which cause deterioration of flavours, colour, texture, appearance and nutritional value, thus aiming to extend the product's shelf life (44). They may have a natural source, such as antimicrobial extracts of plants, or be artificially created, as is the case of weak organic acids (3).

1.2.1.1 Preservatives of Natural Origin

This class of preservatives tends to be well accepted by the consumers, as they circumvent the growing concerns about synthetic chemical additives. A group of promising natural antimicrobials are essential oils from plants, complex mixtures of several bioactive chemical components with antimicrobial activity, like phenolics, phenolic acids and quinones, tannins, terpenoids, glycosides and alkaloids (45). The most relevant are phenolic compounds, whose mechanism of action against microorganisms involves interaction and denaturation of membrane proteins, leading to leakage of ions, other cell content and the eventual cell breakdown (46). Examples of common phytophenols are thymol, p-coumaric acid, chlorogenic acid, gallic acid and caffeic acid, which have demonstrated good antimicrobial and antioxidant activities and potential to be used as natural preservative agents (47, 48). Despite the abundance of plant extract with antimicrobial activities, there

is one limitation to their effective use, as they require higher concentrations to exert a degrading effect on microbiological cells, which is undesirable in foodstuffs as it brings a flavour which overrules over the intrinsic organoleptic characteristics of the food item (49).

1.2.1.2 Artificial Preservatives

Artificial and/or synthetic preservative agents afford a much wider variety of preservation solutions, going beyond those occurring in nature. The most common include saccharin, sorbic acid and its salts (used for food products at higher pH), benzoic acid and its salts (mainly used for acidic food products), parabens (methyl, ethyl, propyl and butylparaben) and salicylic acid (3). One of the main groups of artificial preservatives are weak organic acids, like benzoic acid and sorbic acid, which in aqueous solution partially dissociate, leading to a dynamic equilibrium between molecular acids and charged species. Given that the deprotonated form of these agents is inactive, they should be used in foods with low pH. Furthermore, the hydrophobicity of the non-ionized form allows them to penetrate cells by simple diffusion across the lipidic membrane into the cytoplasm (50). Once inside, their dissociation releases protons, and a high concentration of preservatives will release sufficient protons to cause substantial cytoplasmic acidification, inhibiting cellular metabolism and leading to microbial cell death (51). M. Stratford *et al* recently elucidated the mechanism of sorbic acid inhibition in *S. cerevisiae*, concluding that it works differently, in a mechanism that involves inhibition of the transmembranar H^+-ATPase proton pump (52).

1.3 Antioxidants as Preservation Agents of Food Products

Antioxidant compounds are a promising group of natural and synthetic molecules able to have action as preservatives. They delay the onset and reduce the pace of lipid oxidation reactions, thus being used to extend the shelf life of the product without altering its taste, odour and appearance (53). Antioxidants of natural sources include phenolic acids (*e.g.* caffeic acid and gallic acid), terpenes (*e.g.* pinene), phenolic diterpenes (*e.g.* carnosol), phenylpropanoids (like eugenol) and flavonoids (*e.g.* quercetin and catequin) (54). Synthetic antioxidants are mainly phenolic derivatives that contain various ring substituents (monohydroxy or polyhydroxy phenolic compounds) such as butylated hydroxytoluene (BHT) (3) (Figure 2).

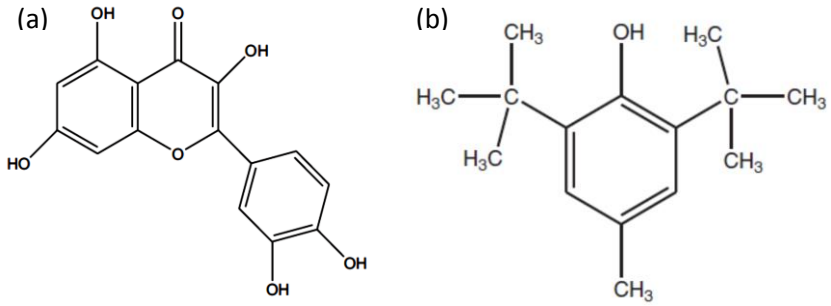


Fig.2 Chemical structures of quercetin (a) and butylated hydroxytoluene (BHT)(b). From (3).

1.3.1 Mechanism of Action of Antioxidants

There are several mechanisms through which antioxidant compounds delay autoxidation: (i) scavenging of species which initiate peroxidation, (ii) chelating of metals to render them unable to generate reactive species or decompose lipid peroxides, (iii) quenching[1] of $O_2^{\bullet -}$ (preventing the formation of peroxides) and/or reduction of localized O_2 concentrations (55). Most of these molecules comprise phenolic rings, and the particular resonance within these makes them very stable, allowing them to scavenge and annihilate free radicals without suffering molecular destabilization (56). Examples of free radical

[1] Quenching is the suppressing or diminishing a physical property. In this case, the quenching of singlet oxygen involves the deactivation of the excited state of the molecule.

scavenging antioxidants are phenolic compounds like tocopherols, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA).

(i) The propensity of a compound to donate electrons to food radicals is predicted by its reduction potential – the lower the reduction potential of the antioxidant radicals, the greater the electron donating ability of the antioxidants. Any compound for which the corresponding radical has a reduction potential lower than the radicals of food or oxygen-related radicals has the ability to act as an antioxidant towards them (21).

(ii) Metal chelators, like EDTA and citric acid, decrease oxidation by preventing the metal redox cycle, forming insoluble metal complexes or providing steric hindrance between metals and food components or their oxidation intermediates (57).

(iii) Singlet oxygen quenching includes both physical and chemical quenching. Physical quenching leads to deactivation of singlet oxygen to the ground state triplet oxygen by energy transfer or charge transfer. Chemical quenching is a reaction involving the oxidation of a quencher rather than quenching, thus producing the breakdown of the quencher and its oxidation products (58). β-Carotene, tocopherols, ascorbic acid, and phenolics are oxidized by singlet oxygen, rendering it inactive and thus having quenching activity (37).

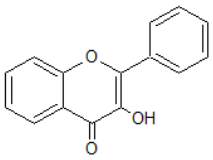
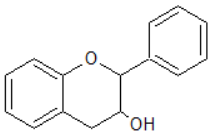
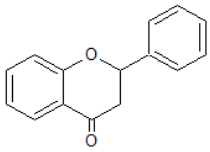
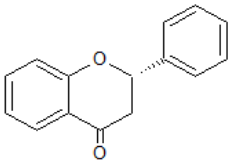
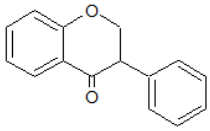
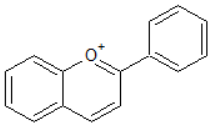
1.3.2 Antioxidant Phenolic Compounds

Phenolic compounds are generally secondary metabolites of plants derived from phenylalanine or tyrosine (59). They include a very broad spectrum of molecules from simple derivatives of benzoic or cinnamic acids, to the complex family of flavonoids and tannins. The major antioxidant plant phenols can be divided into 4 groups: phenolic acids (gallic, protochatechuic, caffeic, and rosmarinic acids), phenolic diterpenes (carnosol and carnosic acid), flavonoids (quercetin and catechin), and volatile oils (eugenol, carvacrol, thymol, and menthol) (60). Antioxidant action may involve trapping or scavenging of free radicals as well as metal chelation (61).

Flavonoids, ubiquitous in plants, are the largest class of polyphenols, with a common structure of diphenylpropanes (C6-C3-C6), consisting of two aromatic rings linked

through three carbons (62). The six major subclasses of flavonoids include the flavones, flavonols, flavanones, flavanols (or catechins), anthocyanidins, and isoflavones (Table 1).

Table 1. Characteristics of the main classes of flavonoids: general structure, examples and sources.

Class	***General structure***	***Examples***	***Sources*** (63)
Flavonol		Quercetin (3,5,7,3',4'-OH) Myricetin (3,5,7,3',4',5'-OH)	Red wine Black Tea Onion leaves Garlic
Flavanol		Catechin (3,5,7,4',5'-OH)	Cocoa Green tea
Flavone		Apigenin (3',4',5,7-OH) Luteolin (4',5,7-OH)	Parsley Celery Carrots Peppers
Flavanones		Naringenin (4',5,7-OH) Hesperetin (4'-OMe; 5,7,5'-OH)	Grapefruit Citrus fruits Dried oregano
Isoflavones		Genistein (4',5,7-OH) Daidzein (4',7-OH)	Leguminous Plants Beans Soy
Anthocyanidins		Cyanidin (3,5,7,3'4'-OH) Malvidin (3,5,7,4'-OH; 3',5'-OMe)	Red Berries Blackberries

Within the subgroups of the flavonols and the flavones, the flavonol quercetin (Figure 2 (a)) is the most frequently occurring compound in foods, with kaempferol, myrcetin, and the flavones apigenin and luteolin also being quite common(64). Dietary sources of quercetin and other flavonoids include blueberries, garlic, onions, kale, broccoli,

spinach, black tea and many other plants (65), with this specific flavonol been widely investigated for its antioxidant capacities. Due to its hydroxylation pattern in carbons 3, 5, 7, 3' and 4', and a cathecol B-ring, quercetin possesses all the structural characteristics for an effective antioxidant, being the most effective one among the flavonols and one of the most effective overall, following epicatechin gallate and epigallocathechin galate (66). Its high capacity to inhibit both metal and non-metal-induced oxidative damage is partially due to its free 3-OH substituents, which are thought to increase the stability of the flavonoid radical(67). This flavonol has also shown protein kinase inhibition capacity, through competition with ATP binding to the nucleotide binding site on the kinases (68), and anti-carginogenic activity through cell cycle arrest at the G2/M transition, by down regulating CDK1 expression and thereby cell proliferation, migration and invasion in androgen independent prostate cancer cells (PC-3) (69). The bioactivity of flavonoids is closely linked to their ability to interact with membranes, since many of these compounds generate a cell response even if they are not internalized. Margina *et al* showed that quercetin and epigallocatechin gallate induce a concentration dependent increase of the membrane anisotropy, and therefore decrease membrane fluidity. By inducing a greater degree of membrane "stiffening" and hyperpolarization, these compounds could reduce the mobility of free radicals in the lipid bilayer, and the decreased membrane fluidity could lead to inhibition of lipid peroxidation(70).

In order to have antioxidant activity, a flavonoid compound must have various structural characteristics that allow it to participate in the redox reactions. As mentioned, the resonance delocalization throughout the phenolic ring modulates the radical scavenging capacity of an antioxidant (Figure 3). So far, three structural arrangements have been identified to provide antioxidant activity (59):

i) An ortho-dihydroxy or cathecol group at the B-ring, which provides a more stable radical form and participates in electron delocalization;

ii) A 2, 3 – double bond, in conjugation with a 4-oxo function in the C-ring, responsible for electron delocalization from the B-ring;

iii) Hydroxyl substitutions at positions 3 and 5, for maximal radical scavenger potential.

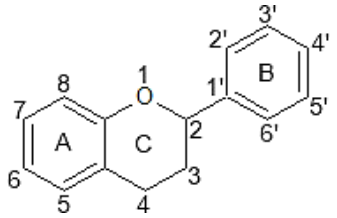


Fig. 3 General structure of a flavonoid phenolic compound.

1.4 Microencapsulation as a Technique to Maintain the Activity of Food Additives

Polyphenols are sensitive compounds, often seen as very unstable and highly susceptible to degradation (71). The stability of polyphenols under different conditions is a very important aspect to consider in the different stages of industrial processing. Inactivation may occur due to aspects as high temperatures, light, oxygen, solvents, the presence of enzymes, proteins, metallic ions, or association with other food constituents (72). Therefore, the administration of phenolic compounds requires its correct formulation into a finished product able to maintain structural integrity until consumption. Ideally, this formulation should also be able to mask the addictive's taste, increase water solubility and bioavailability, and convey these compounds precisely towards the specific physiological target (5). Microencapsulation, developed approximately 60 years ago, is defined as a technology of packaging solids, liquids or gaseous materials in nanoscale, sealed capsules able to release their contents at controlled rates under specific conditions (73). Its two main objectives are to protect the core material from adverse environmental conditions, such as light, moisture and oxygen, thereby contributing to an increase in the shelf life of the product, and to promote a controlled release of the encapsulate (74). In sum, an effective microencapsulation technique should be able to improve the physical, chemical, and biological properties of the food ingredients, to regulate their release and to protect them from damage (75).

Currently, the food industry has a variety of encapsulation techniques at its disposal. Z. Fang and B. Bhandari have summarized the most widely ones applied for polyphenols, which are spray-drying, coacervation, encapsulation into liposomes, molecular inclusion, cocrystallization, freeze drying and nanoencapsulation(76). The molecular inclusion method will be addressed further ahead, for this was the method chosen for this work.

1.4.1 Spray-Drying

Spray-drying is the most widely used microencapsulation technique in the food industry and is typically employed for the preparation of dry, stable food additives and flavours and for the encapsulation of oils and fragrances (74). The process is economical, flexible (in that it offers substantial variation in the microencapsulation matrix) and produces particles of good quality particles (73). The application of the spray-drying process in microencapsulation involves three basic steps: preparation of the dispersion or emulsion to be processed; homogenization of the dispersion; and atomization of the mass into a drying chamber or bed (6). One limitation of the spray-drying technology is the small number of shell materials available. Since almost all spray-drying processes in the food industry are prepared from aqueous feed formulations, the shell material must have a good aqueous solubility(73). N. Fu *et al* have used low-temperature spray drying systems to successfully produce monodisperse epigallocatechin gallate microparticles, which were able to maintain the antioxidant activity of EGCG (77).

1.4.2 Coacervation

The concept behind microencapsulation by coacervation is the phase separation of one or many hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate[2] phase around the active ingredient, suspended or emulsified in the same reaction media (78). The complex coacervation method principally consists in an emulsification, which creates an oil-in-water emulsion, coacervation of the oil droplet surface by adjusting the pH of the solution, and fixation of the shell membrane by gelation of the coacervates or by chemical crosslinking (79). Despite the intrinsic advantage and unique properties of this method when compared to other common encapsulation processes, coacervated food ingredients have low commercial acceptance due to the very

[2] A coacervate is a tiny spherical droplet of assorted organic molecules (specifically, lipid molecules) which is held together by hydrophobic forces from a surrounding liquid. They range generally in size from 1 to 100 µm, possess osmotic properties and form spontaneously from certain dilute organic solutions. Their name derives from the Latin word coacervate, meaning “to assemble together or cluster” (169).

high cost and complexity of the production process. In addition, crosslinking of the shell material usually involves glutaraldehyde, which must be carefully used according to the country's legislation, for it has toxic and carcinogenic properties (80). However, the processing cost can be dramatically decreased by optimizing the isolation procedure at the end of the encapsulation step (81). M.G. Santos *et al* have produced microcapsules of xylitol with desirable characteristics for use in foods (particle size below 109 μm, low solubility and complete encapsulation of the core by the wall material), in order to control the release of this ingredient and prolong its sweetness and freshness when applied in foods such as chewing gums (82).

1.4.3 Liposome Microencapsulation

A liposome is an artificially-prepared spherical vesicle composed of a lamellar phase lipid bilayer. It is formed when the lipid molecules that compose the lipid bilayer are dispersed in aqueous media and exposed, through disruption of the biological membranes (*e.g.* by sonication). The underlying mechanism for the formation of liposomes is based on the hydrophilic–hydrophobic interactions between the lipid and water molecules (83), that can be used in the entrapment, delivery, and release of water soluble, lipid-soluble, and amphiphilic materials. The great advantage of liposomes over other microencapsulation technologies is the stability that they impart to water-soluble materials in high water activity environments and the targeted delivery of their content in specific parts of the foodstuff. The main issues for the food industry when using liposomes are the scaling up of the microencapsulation process at acceptable cost-in-use levels and the delivery form of the liposome-encapsulated ingredients. In fact, these formulations are kept in relatively dilute aqueous suspensions, which poses serious concerns to the large-scale production, storage, and shipping of encapsulated food ingredients (73). B.D. Isailović *et al* have investigated the incorporation of resveratrol in liposomes with the aim of developing an easily upscalable process for liposome production (84).

1.4.4 Co-Crystallization

Co-crystallization is an encapsulation process in which the crystalline structure of the compound is modified from a perfect to an irregular agglomerated crystal, thus providing a porous matrix in which a second active ingredient can be incorporated (85). The main advantages of this method are the improved solubility, wettability, homogeneity, dispersibility, hydration, anticaking, stability and flowability of the encapsulated materials (86), and this technique offers an economic and flexible alternative for the incorporation of active compounds into powder foods. A. López-Córdoba *et al* have obtained yerba mate antioxidant powders with high entrapment yield through co-crystallization, and the process allowed to maintain the antioxidant activity of the yerba mate extract largely unaltered. The obtained powders showed suitable characteristics such as low water content and water activity, high solubility, low hygroscopicity and very good flowability, thus constituting a promising alternative as a natural antioxidant ingredient for the formulation of functional foods (87).

1.4.5 Freeze Drying

Freeze drying, also known as lyophilization or cryodesiccation, is a process used for the dehydration of almost all heat-sensitive materials and aromas. The process of encapsulation has generally two steps, starting with the mixing of the core molecule in a coating solution followed by freeze drying of the resultant mixture (73). Except for the long dehydration period required (generally 20 h), freeze-drying is a simple technique for trapping water-soluble essences and natural aromas, as well as drugs (73). After freezing the mixture, reducing the surrounding pressure and adding enough heat, the frozen water present will sublimate directly from the solid phase to the gas phase, creating a dry material composed of coated core material (88). M.R. Pérez-Gregorio *et al* studied the effects of freeze-drying on the flavonoid content of onions, and verified that the process facilitated their extraction, possibly due to the changes it induces in the structure of onion tissues. This technique also showed to be useful application in the production of onion powder for cooking, retaining the antioxidant properties of the product for six months (89).

1.4.6 Nanoencapsulation

Nanoencapsulation involves the formation of active loaded particles with diameters ranging from 1 to 1000 nm (90). Compared to micron-sized particles, nanoparticles provide a greater surface area and have the potential to increase solubility due to a combination of large interfacial adsorption of the core compound, enhanced bioavailability and improved controlled release, which enable better precision in targeting of the encapsulated materials (74). F. Donsì *et al* investigated the nanoencapsulation of two essential oils from plants rich in terpenes, using soy lecithin, modified starch (CLEARGUM) and a mixture (50:50) of Tween 20 and glycerol monooleate as emulsifiers. They verified that the terpene-loaded nanocapsules were able to delay or completely inactivate microorganisms present in fruit juices, while minimally altering their organoleptic properties (91).

1.4.7 Overview and Future Perspectives for Encapsulation Techniques in Food

When choosing a method for encapsulation, there are several factors that must be taken into account. Besides the obvious requirement for protection of the bioactive component against any loss of activity during the whole period of processing, storage, and transport(92), it is also desirable that the encapsulation system allows an efficient package load (which depends on the type of bioactive molecule and specific vehicle (93), and an easy incorporation into the food product without interfering with its texture and taste, meaning that the delivery system should be compatible with the surrounding food matrix (94). In addition, the delivery system must control the bioactivity or bioavailability of the encapsulated compounds (95).

Regarding the future research of encapsulation, improvements in manufacturing technologies, new strategies for stabilization and discovery of novel approaches to site-specific carrier targeting will play an important role in increasing the efficacy of encapsulating techniques over the next decade (76). When compared to the wide range of encapsulated products already developed, manufactured and successfully marketed in the pharmaceutical and cosmetic industries, the penetration of microencapsulated products into the food industry is rather limited, for the development time is still long and requires

a multidisciplinary cooperation. The most important aspect is an understanding of the industrial constraints and requirements to make a microencapsulation process viable, from the transition to full-scale production to the marketing of the final product (73).

In this work, the microencapsulation of the flavonol quercetin was carried out by molecular inclusion with cyclodextrins, and the following subsection is an exposition of these host molecules and of their role in the process.

1.5 Cyclodextrins as Agents of Molecular Encapsulation

Cyclodextrins (CDs) are a family of cyclic oligosaccharides composed by glucopyranose units, being crystalline, homogenous and nonhygroscopic substances shaped like a trunked cone, in a torus-like macro-ring sctructure (96). The three major cyclodextrins are α, β and γ-cyclodextrin (α-CD, β-CD and γ-CD), which are composed by six, seven and eight glucopyranose units, respectively (Figure 4). The most important chemical characteristics of these compounds are summarized in Table 2 (96).

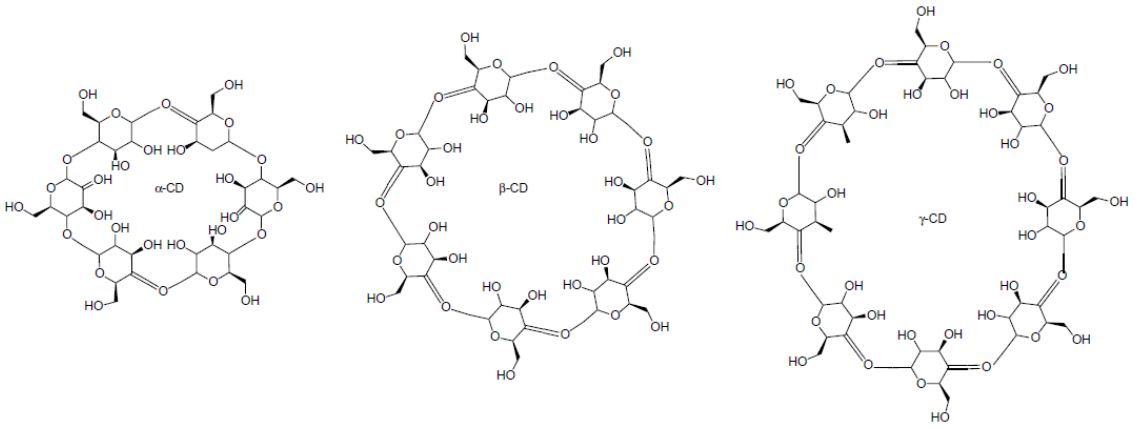


Fig. 4- The various structures of CDs ((97)Bilensoy E, editor. Cyclodextrins in Pharmaceutics, Cosmetics, and Biomedicine).

Table 2- General characteristics of major CDs (adapted from (96)).

	α	β	γ
Number of glucose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water, g 100mL^{-1} at room temperature	14.5	1.85	23.2
Cavity diameter, Å	4.7-5.3	6.0-6.5	7.5-8.3
Approximate volume of cavity, Å^3	174	262	427

Similar to amylose, the glucose units in cyclodextrins are linked by α(1-4) bonds and adopt the $^4C_1$ chair conformation (Figure 5), and the distribution of hydrophilic and hydrophobic groups is very important. Both rims of the cyclodextrin's cone are occupied by hydroxyl groups, which renders them soluble into water, while their cavity has a hydrophobic character conveyed by the C3-H, C5-H, and C6-H_2 hydrogens and by the ether-

like oxygens O4 (98). This combination of a hydrophobic micro-environment able to exist within an aqueous solution gives cyclodextrins a great versatility of use in multiple applications.

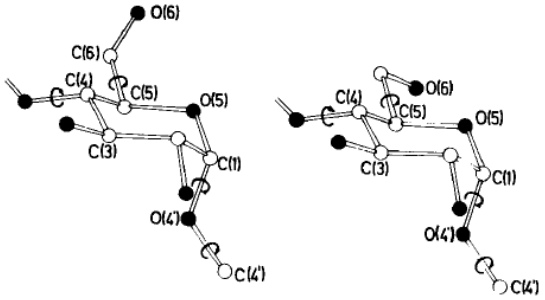


Fig. 5 - Glucose $^4C_1$ chair conformation with O6-H in (-)gauche (left) and (+)gauche (right) orientations. The arrows in circles represent bond rotations (97).

1.5.1 Brief History of Cyclodextrins

Cyclodextrins were first discovered in the late 19th century by a pharmacist and chemist named Antoine Villiers. In his work, entitled "*Sur la fermentation de la fécule par l'action du ferment butyrique*", he mentions that potato starch can ferment under the action of the strain of bacteria *Bacillus amylobacter*, producing fermentation products predominantly composed by dextrins and, at the same time, a carbohydrate that would separate into circular crystals after some weeks, in the alcohol used to precipitate the dextrins (99). He proceeded into denominating this new carbohydrate as *cellulosine*, in order to differentiate it from saccharines.

After their discovery by Villiers, the study of cyclodextrins was approached by Franz Schardinger, who was investigating a type of extremely heat resistant microorganism which revealed able to dissolve starch and form crystalline products similar to the ones reported by Villiers (100). He then proceeded to thoroughly study these carbohydrates, giving the first detailed description of their preparation, separation and purification and deciding to name them crystalline α-dextrin and β-dextrin (101). Schardinger became known as the "Founding Father" of cyclodextrin chemistry, being considered the first to describe their fundamental properties and their ability to form complexes.

Following the works of Villiers and Schardinger, the knowledge of cyclodextrins entered the so called "period of doubt", from 1911 to 1935 (102). During this time, their structure was not yet defined, and the works developed would focus mainly in the premise of Shardinger's dextrins being able to further help understand the synthesis and degradation of starch. One researcher that stood out in this period was Hans Pringsheim, who elaborated an interpretation of a possible structure for amylose and amylopectin from

Schardinger's observations (103), and worked in the isolation of α and β-dextrins, confirming Schardinger results (104).

It was only from 1935 onwards that the study of cyclodextrins registered great developments. Karl Johann Freudenberg and his team described a method of synthesis of high purity dextrins, and determined, even though incorrectly, the number of glucose units that composed them (105). Freudenberg is acknowledged for the determination of the cyclic structure of α and β-dextrins (106), and was the first to propose the mechanism of action of *B. macerans*. (107). Another great contributor for the knowledge of cyclodextrins was Dexter French who, together with Robert E. Rundle, elucidated the correct molecular weight and the exact number of glucose units of α and β-dextrins, through X-Ray diffraction and crystal density measurements (108).

After the determinations of the chemical properties of cyclodextrins, it was time for their study to enter a period of concern around their applications. Friedrich Cramer showed that the main value of cyclodextrins resided in their ring structure and ability to include guest molecules inside them and explained the mechanisms for inclusion and creation of complexes in solution and in the solid state (109). He also contributed in the elucidation of various structural and physico-chemical details of cyclodextrins, such as cavity size, solubility and reactivity (110).

It would be impossible to do a complete historical overview of the development of cyclodextrin science without mentioning the work of József Szejtli, considered to be the "Godfather of Cyclodextrins" (102). He studied and reformulated the interpretations on the mechanisms of formation of inclusion complexes made by Cramer and others, adding some important details and new concepts (111, 112), and also established that the application of cyclodextrins would mainly take advantage of the effects caused by the inclusion process in the guest molecule (113). Szejtli had a very important role in the dissemination of cyclodextrin knowledge, through the publication of various reviews and books on the topic (114, 115), and also by the creation of a company completely dedicated to the study of cyclodextrins, the CYCLOLAB Ltd. (116).

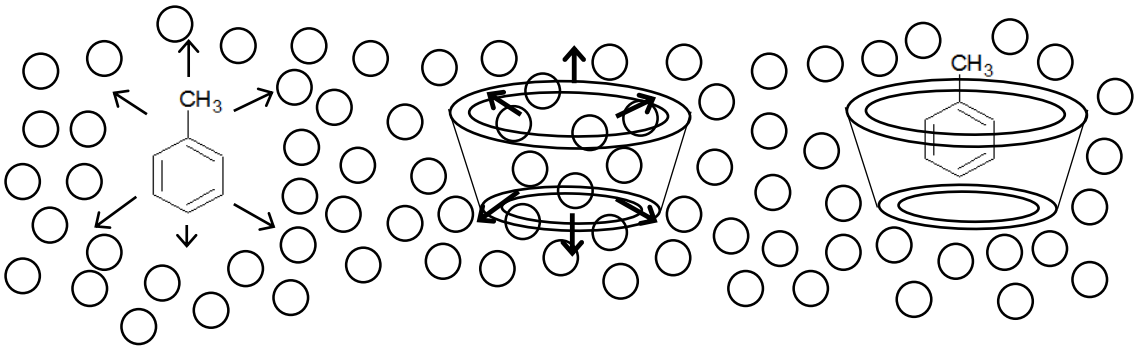
1.5.2 Synthesis of Cyclodextrins

Cyclodextrins are obtained by various biochemical transformations applied to starch, such as hydrolysis and enzymatic conversions. First, the starch is liquefied at high temperatures, to allow its partial depolymerisation and facilitate the subsequent enzymatic processes (117). Then, the enzyme cyclodextrin glycosyl transferase (1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano) -transferase, E.C. 2.4.1.19, CGTase) is added to catalyse various transglycosilation reactions, such as intramolecular cyclisation, intermolecular coupling reaction (creating various open chain oligosaccharides), among others (118). This enzyme can be produced by various strands of bacteria, but its source is usually a culture of *Bacillus macerans* (119). After the addition of CGTase, the production of CDs can be achieved by two distinct processes: "solvent-process" and "non-solvent process" (120). In the solvent-process, a complexing agent is added to the previous solution, promoting the selective precipitation of one type of CD and directing the enzyme reactions towards this type of CD. The non-solvent processes do not involve the addition of complexating agents, having the advantage of avoiding the use of organic solvents but also the disadvantage of having to purify the product obtained, which is composed by a mixture of CDs (121). The ratio of CDs produced will depend on the CGTase used (which, in its turn, depends on the strain of bacteria selected) and on the reaction conditions, such as pH, temperature and reaction time (122).

1.5.3 Cyclodextrins and the Formation of Inclusion Complexes

Cyclodextrins are able to form inclusion complexes with guest molecules, both in solution as in solid state. In solution, they can be formed by simply dissolving together the cyclodextrin and the desired guest molecule, a type of complex that involves only intermolecular interactions. Crystalline inclusion compounds may be prepared by crystallization from the guest solvent or by cocrystalization of host and guest molecules from an inert solvent. Solid complexes can also sometimes be prepared by grinding solid host and guest together (123). The formation of the inclusion complex is a dynamic equilibrium process consisting in the substitution of the included water molecules by the

less polar guest, which is energetically favoured by the interaction of the guest molecule with the solvated hydrophobic cavity of the cyclodextrin (124) (Scheme 3).



Scheme 3-Formation of a cyclodextrin-toluene inclusion complex

The formation of the inclusion complex affects both the host and the guest molecule. In solution, the spectral properties of the guest are modified, and its reactivity, diffusion and volatility decreases. Its hydrophilicity increases, and the inclusion complex is soluble in water. In the solid state, the included molecule is effectively protected from external reactions, and sublimation and volatility are also reduced to a very low level (96, 125).

1.5.4 Applications of cyclodextrins

After the long period of discovery, cyclodextrins were studied for their possible industrial application, in a so called “Period of application” which started around 1970 and lasts until now (102). Before this, there were many questions about the safety of the human consumption of cyclodextrins, and they were deemed toxic for some time (126). However, with following studies, it was considered that “any toxic effect is of secondary character and can be eliminated by selecting the appropriate CD type or derivative or mode of application” (96), and cyclodextrins were considered to be safe for human consumption. Table 3 summarizes the regulatory status of native cyclodextrins.

Table 3- Regulatory status of native cyclodextrins(127).

Product	*Type IV DMF No.*	*Food Approval*			*Pharmacopeia Monographs*		
		US	*Europe*	*Japan*	*USP/NF*	*Ph. Europe*	*Japan*
α-cyclodextrin	14937	GRAS	Novel Food 2008	Yes	Yes (Alfadex)	Yes	Yes
β-cyclodextrin	14620	GRAS	Food Additive	Yes	Yes (Betadex)	Yes	Yes
γ-cyclodextrin	11375	GRAS	N/A	Yes	Draft published	In process	No

The approval of cyclodextrins as "Generally Regarded As Safe" (GRAS) has led to a booming growth in the publications and in the applications of cyclodextrins in many fields such as pharmaceutical, analytical, alimentary, cosmetic, textile and so on.

The reason for such an interest in cyclodextrins arises mainly from their ability to form inclusion complexes with other molecules in a selective way, being able to perform molecular and chiral recognition (128), and from the advantages associated with the encapsulation of the guest molecule, such as modifications of its physicochemical properties and activity, stabilization against degradation by light and oxygen, volatilily reduction and many others (113).

1.5.4.1 Cyclodextrins in the pharmaceutical industry

Cyclodextrins are used in pharmaceutical formulations mainly as agents for solubilisation and stabilization, enhancers of bioavailability, and as drug delivery systems (97). The physicochemical characteristics of most drugs, such as low polarity, low to medium molecular mass and the presence of aromatic structures, makes them ideal guest candidates, able to get included in the cavity of cyclodextrins and to take advantage of the benefits of such inclusion (96). The hydrophobicity and consequent slow and incomplete absorption of most drugs is circumvented by cyclodextrin inclusion (96); compounds which are sensitive to external factors such as oxygen, temperature and light, suffering easily oxidation and decomposition, are also stabilised upon inclusion, allowing for the drug to be transported safely to its active site and exert its action. Table 4 presents some cyclodextrin –containing drugs already in the market.

Table 4-Drugs with cyclodextrins in their formulation (129).

Drug	*Trade name*	*Formulation*	*Company (country)*
α-Cyclodextrin			
Alprostadil	Caverject Dual	Intravenous solution	Pfizer (Europe)
Cefotiam-hexetil HCl	Pansporin T	Tablet	Takeda (Japan)
Limaprost	Opalmon	Tablet	Ono (Japan)
β-Cyclodextrin			
Cephalosporin	Meiact	Tablet	Meiji Seika (Japan)
Cetirzine	Cetrizin	Chewable tablet	Losan Pharma (Germany)
Chlordiazepoxide	Transillium	Tablet	Gador (Argentina)
Nicotine	Nicorette	Sublingual tablet	Pfizer (Europe)
Nimesulide	Nimedex	Tablets	Novartis (Europe)
Nitroglycerin	Nitropen	Sublingual tablet	Nihon Kayaku (Japan)
Omeprazole	Omebeta	Tablet	Betafarm (Europe)
2-Hydroxypropyl-β-Cyclodextrin			
Cisapride	Propulsid	Suppository	Janssen (Europe)
Indometacin	Indocid	Eye drop solution	Chauvin (Europe)
Itraconazole	Sporanox	Oral and intravenous solution	Janssen (Europe, USA)
Mitomycin	MitoExtra, Mitozytrex	Intravenous infusion	Novartis (Europe)
Sulfobutylether β-Cyclodextrin sodium salt			
Aripiprazole	Abilify	Intramuscular solution	Bristol-Myers Squibb (USA); Otsuka Pharm. (USA)
Maropitant	Cerenia	Parenteral solution	Pfizer Animal Health (USA)
Voriconazole	Vfend	Intravenous solution	Pfizer (USA, Europe, Japan)
Ziprasidone mesylate	Geodon, Zeldox	Intramuscular solution	Pfizer (USA, Europe)

Table 4 (cont.)-Drugs with cyclodextrins in their formulation (129).

Drug	*Trade name*	*Formulation*	*Company (country)*
Randomly methylated β-cyclodextrin			
17β-Estradiol	Aerodiol	Nasal spray	Servier (Europe)
Chloramphenicol	Clorocil	Eye drop solution	Oftalder (Europe)
2-Hydroxypropyl-γ-cyclodextrin			
Diclofenac sodium salt	Voltaren Ophtha	Eye drop solution	Novartis (Europe)
Tc-99 Teboroxime	CardioTec	Intravenous solution	Bracco (USA)

1.5.4.2 Cyclodextrins in Textiles

The permanent fixation of supramolecular compounds, such as cyclodextrins, to the surface of textiles allows the achievement of new functional properties, through selective inclusion of other chemical species into the fibre (130). Cyclodextrins are able to form inclusion complexes with surfactant molecules and also act as cleaning agents, being very useful for the removal of detergents in washed textiles and in reducing the volume of water needed for rinsing (131).

The dyeing of textiles can be enhanced by applying cyclodextrins on their surface, for they can encapsulate the dye particles and facilitate the process by other interactions such as acid-base, hydrogen bonding or even covalent bonding, making it more effective and environmentally friendly (132). Cyclodextrins can also be used as textile fresheners due to their ability to include odour molecules, removing unpleasant smells, or conveying perfuming agents (133). Other uses of CDs in the textile industry include the encapsulation of insect repellents and antimicrobial agents, enhancement of cord strength of polyester fibers and also the control of wrinkles in clothes (134).

1.5.4.3 Cyclodextrins in Analytical Techniques

The analytical applications of cyclodextrins are based on the reversible interaction between the cyclodextrin and the guest molecule (135). The selective character of the

inclusion process gives them great utility in analytical techniques of spectroscopy, separation and detection.

Cyclodextrins can form inclusion complexes with target analytes and interfering components of a matrix from complex analytical samples, which makes them useful for sample preparation. This decreases inferences from the matrix effects, improving the accuracy and reliability of common analytical techniques (136). They can also improve sensitivity through a significant and temporary change in the microenvironment of the analyte in their cavity, which results in a spectral response that allows for an easier detection (137). Another analytical field that takes advantage from selective inclusion complexes and where cyclodextrins are widely applied are separation techniques, such as chromatographic separations, HPLC (High-performance liquid chromatography) and capillary electrophoresis (135).

1.5.4.4 Cyclodextrins in the Food Industry

Cyclodextrins were applied in the food industry very early on. They are considered natural products in Japan, and their application occurred right after the development of industrial manufacturing processes for production of cyclodextrins, from the late 1970's to early 1980's (138). In 1987, there were already 88 Japanese patents for application of cyclodextrins in food products, making Japan the front-runner in having foods functionalized with cyclodextrins. The approval in the United States came much later, with γ-cyclodextrin being the first to be approved in 2000 and followed by β-cyclodextrin in 2001 and α-cyclodextrin in 2004 (139 – 141). In Europe, cyclodextrins α, β and γ have all been approved for use in food products and commercialization by the European Commission as of 2012 (142 – 144).

Cyclodextrins are of great interest for alimentary uses, for they can bring solutions to various problems that appear in food processing and product development. In general, cyclodextrins are used as food additives as: (i) protectors of food components that are sensitive to light, oxygen and heat; (ii) solubilising agents for food colouring and lipophilic vitamins; (iii) stabilisers of fragrances, flavours, vitamins and essential oils; (iv) suppressants

of unpleasant odours and tastes and (v) agents for controlled release of certain food constituents (124).

The ability to form inclusion complexes gives cyclodextrins a multitude of applications in the food industry that take advantage of this phenomenon. As mentioned, they are used to encapsulate volatile and sensible compounds, whose complexes can then be used to protect such compounds from degradation (145) and also include them in food formulations as enhancers (146), to remove or mask unpleasant flavours and smells (147) and to stabilise oils (148) and retain oil volatiles (149). Cyclodextrins are also used as stabilisers of oil-in-water emulsions, being used as emulsifiers for salad dressings, margarine and mayonnaise (150).

Cyclodextrins can also act as sequestrants of food components, being effectively used to remove cholesterol from various dairy products such as milk (151), butter (152), cheese (153) and cream (154). They were also studied as anti-browning agents able to inhibit the action of polyphenol oxydase (PPO), the enzyme responsible for the enzymatic browning of food. They have been applied in fruit juices as “secondary antioxidants”, to preserve their colour and prolong shelf life (155, 156), and the method of use of cyclodextrins as preservants of raw fruits and vegetables juices has been patented (157). Table 5 presents some marketed foods that contain CDs.

Table 5-Food products that include cyclodextrins in their formulations (158).

Trade name	***Type of food product***	***Function of CDs used***
Natual (France)	Low cholesterol cheese	To reduce cholesterol
Cyroma-line (Hungary)	Flavored sugar for baking	To preserve flavour on heating
Balade (Belgium)	Low cholestrol butter	To reduce cholestrol
Simply Eggs (USA)	Low cholestrol egg	To reduce cholestrol
FlavorAktiv Standard Kit (Great Britain)	Beer flavour standards	To preserve flavour standards
Flavono (Japan)	Chewing gum	To stabilize flavour
Choco Bar (Japan)	Chocolate	For emulsification
Poder Tea (Japan)	Instant green tea	For stabilization of colour
Gymet (Japan)	Dietary fibre drink	For taste masking
Stick Lemon (Japan)	Instant tea drink	To preserve flavour

Another use for cyclodextrins in the food industry is in food packaging materials. They can be for microbial safety, through the complexation with antiseptic and antimicrobial guests and then applying such complexes on the surface of the package (159), and also for preservation, through the formation of inclusion complexes with antioxidant and preservative guest molecules (160).

1.6 Purpose of the Work

Currently, there is a plethora of well-developed encapsulation techniques with widespread use in the pharmaceutical industry, with a strong economical supremacy. However, their practical utility in the food industry is limited, thus raising the need for innovative solutions. The nutraceutical and functional food market should not be ignored, as it shows a great potential for growth, being expected to grow above US$243 billion in 2015. According to a report by Leatherhead Food Research, the global functional food market is forecast to reach $54 bn (€43 bn) by 2017, an 25% increase compared to the last available data from 2013 (161).

Research in this field is thus on the spotlight, and the newly developed methods and technologies will bring a strong impulse for further studies. The food preservation area has evolved immensely, from the simple salting of foods to the design of complex encapsulated preservatives, and it will continue to develop with the investigation and understanding of the biological mechanisms responsible for food deterioration, as well as the discovery of new compounds capable of inhibiting such processes. Even though there are reports on encapsulated phenolic compounds, most of them are applied to the clinical field (162). Quercetin, the most researched and well-characterized antioxidant compound, features in a vast number of reports regarding its encapsulation in several agents (157-160), the great majority, if not all, targeting pharmaceutical applications. However, due to its poorly aqueous solubility and instability in physiological medium, quercetin has poor bioavailability, poor permeability, instability and an extensive first pass metabolism before it reaches the systemic circulation (164). A shift in the application field of these quercetin-conjugates towards food applications is, therefore, promising and expected to have a good outcome, since its ability to preserve food is an obvious consequence of its antioxidant action, while it is well tolerated by the human organism.

The projected work for this Master thesis in Biochemistry aims at evaluating the potential of using quercetin as a food preservative. Quercetin was chosen considering its well-known antimicrobial (165), metal-chelating (166) and antioxidant (167) properties, which are beneficial for interactions with food deteriorating mechanisms. It will be encapsulated at the molecular level with cyclodextrins. This method was chosen due to the

advantages it brings to the desired product. Cyclodextrins have a wide application in the food industry (124), and apart from being encapsulating agents, they are good molecular carriers, being fairly bioadaptable and hardly absorbable from the gastrointestinal tract. Cyclodextrins resist the low pH of the stomach, thus being likely stable in low pH food products, retaining the carrier effect and allowing the compound to be active in acidic environment, a situation already verified by Zhao M *et al* (168). They also display some technological advantages that include the formation of stable and standard formulations, the simplicity in dosing and handling their dry powders, and the reduced packaging and storage costs (112). The vast application of cyclodextrins as food ingredients has been thoroughly reviewed by Lajos Szente and Jozsef Szejtli (158).

Inclusion of quercetin in cyclodextrins β and γ is expected to preserve its activity and extend its period of action. It may also decrease the dose necessary to exert antioxidant activity. This inclusion complex between quercetin and the cyclodextrin will be characterized (through spectroscopic techniques) for a further understanding of possible structure-activity relationships (SARs), tested for antioxidant activity and incorporated in a milk product (fresh cheese). As a final goal, it is expected to be fulfilled the existing void regarding applications of quercetin complexes as food preservatives and thus contributing towards the understanding of antioxidant properties and mechanisms of cyclodextrin-included flavonoids, either alone or incorporated in food formulations.

References

1. Fellows P, Axtell B. Appropriate Food Packaging. Amsterdam: Tool Publications; 1993.
2. Dauthy ME. Fruit and vegetable processing. Rome: FAO Agricultural Services Bulletin No.119; 1995.
3. Msagati TAM. Chemistry of Food Additives and Preservatives. Oxford, UK: Blackwell Publishing Ltd.; 2012.
4. André C, Castanheira I, Cruz JM, Paseiro P, Sanches-Silva A. Analytical strategies to evaluate antioxidants in food: a review. Trends Food Sci Technol. 2010;21(5):229–46.

5. Munin A, Edwards-Lévy F. Encapsulation of natural polyphenolic compounds - a review. Pharmaceutics. 2011; 3(4), 793-829.

6. Gharsallaoui A, Roudaut G, Chambin O, Voilley A, Saurel R. Applications of spray-drying in microencapsulation of food ingredients: An overview. Food Res Int. 2007; 40(9):1107–21.

7. Barth M, Hankinson TR, Zhuang H, Breidt F. Compendium of the Microbiological Spoilage of Foods and Beverages. Sperber WH, Doyle MP, editors. New York, NY: Springer New York; 2010.

8. Gram L, Ravn L, Rasch M, Bruhn JB, Christensen AB, Givskov M. Food spoilage—interactions between food spoilage bacteria. Int J Food Microbiol . 2002; 78(1-2):79–97.

9. Jacxsens L, Devlieghere F, Ragaert P, Vanneste E, Debevere J. Relation between microbiological quality, metabolite production and sensory quality of equilibrium modified atmosphere packaged fresh-cut produce. Int J Food Microbiol. 2003; 83(3):263–80.

10. Arslan S, Eyi A, Özdemir F. Spoilage potentials and antimicrobial resistance of Pseudomonas spp. isolated from cheeses. J Dairy Sci. 2011; 94(12):5851–6.

11. Baylis CL. Enterobacteriaceae. In: Blackburn C, editor. Food Spoilage Microorganisms. Cambridge, UK: Woodhead Publishing Ltd; 2006; 624–67.

12. Bigelis R. Food enzymes. In: Finkelstein, BC, editor. Biotechnology of Filamentous Fungi Technology and Products. Boston: Butterworth-Heinemann; 1992. p. 361-415.

13. Illy A, Viani R, editors. Espresso Coffee: The Science of Quality. San Diego: Academic Press Inc.; 1995.

14. Filtenborg O, Frisvad JC, Thrane U. Moulds in food spoilage. Int J Food Microbiol. 1996; 33(1):85–102.

15. Legras J-L, Merdinoglu D, Cornuet J-M, Karst F. Bread, beer and wine: Saccharomyces cerevisiae diversity reflects human history. Mol Ecol. 2007; 16(10):2091–102.

16. Loureiro V. Spoilage yeasts in the wine industry. Int J Food Microbiol. 2003; 86(1-2):23–50.

17. Luque MI, Córdoba JJ, Rodríguez A, Núñez F, Andrade MJ. Development of a PCR protocol to detect ochratoxin A producing moulds in food products. Food Control. 2013; 29(1):270–8.

18. Pires ACDS, Soares NDFF, da Silva LHM, da Silva MDCH, De Almeida MV, Le Hyaric M, *et al*. A colorimetric biosensor for the detection of foodborne bacteria. Sensors Actuators B Chem. 2011; 153(1):17–23.

19. Maturano YP, Mestre MV, Esteve-Zarzoso B, Nally MC, Lerena MC, Toro ME, *et al*. Yeast population dynamics during prefermentative cold soak of Cabernet Sauvignon and Malbec wines. Int J Food Microbiol. 2015; 199:23–32.

20. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem. 2005; 53(10):4290–302.

21. Choe E, Min DB. Chemistry and reactions of reactive oxygen species in foods. Crit Rev Food Sci Nutr. 2006; 46(1):1–22.

22. Shacter E. Quantification and significance of protein oxidation in biological samples. Drug Metab Rev. 2000; 32:307–26.

23. Min DB, Choe EO. Effects of singlet oxygen oxidation on the flavor of foods and stability of vitamins. Food Sci Biotechnol. 2002; 11:582– 586.

24. Davies MJ, Dean RT, editors. Radical-mediated Protein Oxidation: From Chemistry to Medicine. Oxford, UK: Oxford University Press; 1997.

25. Lund MN, Heinonen M, Baron CP, Estévez M. Protein oxidation in muscle foods: a review. Mol Nutr Food Res. 2011; 55(1):83–95.

26. Mercier Y, Marinova P, Tassy C, Gatellier P, Renerre M, Recherches S De, *et al*. Comparison of Oxidative Processes on Myofibrillar Proteins from Beef during Maturation and by Different Model Oxidation Systems. 1997; 2481–7.

27. Ndersen HEJA. Identification of Specific Oxidatively Modified Proteins in Chicken Muscles Using a Combined Immunologic and Proteomic Approach. 2004;

28. Aron CAPB, Essen FLJ. Identification of carbonylated protein in frozen rainbow trout (Oncorhynchus mykiss) fillets and development of protein oxidation during frozen storage. J Agric Food Chem. 2006; 54(25):9437-46.

29. Fenaille F, Parisod V, Tabet J-C, Guy PA. Carbonylation of milk powder proteins as a consequence of processing conditions. Proteomics. 2005; 5(12):3097–104.

30. Zakrys-Waliwander PI, O'Sullivan MG, O'Neill EE, Kerry JP. The effects of high oxygen modified atmosphere packaging on protein oxidation of bovine M. longissimus dorsi muscle during chilled storage. Food Chem. 2012; 131(2):527–32.

31. Traore S, Aubry L, Gatellier P, Przybylski W, Jaworska D, Kajak-Siemaszko K, *et al*. Effect of heat treatment on protein oxidation in pig meat. Meat Sci. 2012; 91(1):14–21.

32. Hamilton RJ, Kalu C, Prisk E, Padleyb FB, Pierceb H. Chemistry of free radicals in lipids. Food Chem. 1997; 60(2):193–9.

33. Wasowicz E, Gramza A, Hêœ M, Jeleñ HH, Korczak J, Malecka M, Mildner-Szkudlarz S, Rudziñska M, Samotyja U, Zawirska-Wojtasiak R. Oxidation of lipids in food. Pol J Food Nutr Sci 2004; 13:87-100.

34. Frankel EN. Photooxidation of unsaturated fats. In: Frankel EN, editor. Lipid Oxidation. Scotland: The Oily Press; 1998. p. 43–54.

35. Choe E, Min DB. Chemistry of deep-fat frying oils. J Food Sci . 2007; 72(5):77–86.

36. Grosch W. Lipids degradation products and flavours. In: Morton ID, Macleod AJ, editors. Food Flavours Part A. Amsterdam: Elsevier Scientific Publishing Company; 1982. p. 325–98.

36. Brash AR, Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J Biol Chem. 1999;274(34):23679-82.

37. Choe E, Min DB. Mechanisms of Antioxidants in the Oxidation of Foods. Compr Rev Food Sci F. 2015; 8:345–58.

38. Grosch W. Lipids degradation products and flavours. In: Morton ID, Macleod AJ, editors. Food Flavours Part A. Amsterdam: Elsevier Scientific Publishing Company; 1982. p. 325–98.

39. Pokorny J, Yanishlieva N, Gordon M., editors. Antioxidants in food - Practical Applications. Cambridge, UK: Woodhead Publishing Limited; 2001.

40. Hidalgo FJ, Navarro JL, Delgado RM, Zamora R. Histamine formation by lipid oxidation products. Food Res Int. 2013 ;52(1):206–13.

41. Ibargoitia ML, Sopelana P, Guillén MD. 1H Nuclear Magnetic Resonance monitoring of the degradation of margarines of varied compositions when heated to high temperature. Food Chem. 2014 ;165:119–28.

42. Carocho M, Barreiro MF, Morales P, Ferreira ICFR. Adding Molecules to Food, Pros and Cons: A Review on Synthetic and Natural Food Additives. Compr Rev Food Sci Food Saf. 2014; 13(4):377–99.

43. Vaz MIPM, Mendonc DIMD De, Mendonc JG De. Activity coefficients in the evaluation of food preservatives. Innov Food Sci Emerg Technol. 2001; 2(3): 175–9.

44. Batt CA, Robinson RK, editors. Encyclopedia of Food Microbiology. 2nd ed. Waltham: Academic Press Inc.; 2014.

45. Voon HC, Bhat R, Rusul G. Flower Extracts and Their Essential Oils as Potential Antimicrobial Agents for Food Uses and Pharmaceutical Applications. Compr Rev Food Sci Food Saf. 2012; 11(1):34–55.

46. Nychas G-JE, Skandamis PN, Tassou CC. Antimicrobials from herbs and spices. In: Roller S, editor. Natural Antimicrobials for the Minimal Processing of Foods. Cambridge, UK: Woodhead Publishing Limited; 2003. p. 176-200.

47. Medini F, Fellah H, Ksouri R, Abdelly C. Total phenolic, flavonoid and tannin contents and antioxidant and antimicrobial activities of organic extracts of shoots of the plant Limonium delicatulum. J Taibah Univ Sci. 2014; 8(3):216–24.

48. Govardhan Singh RS, Negi PS, Radha C. Phenolic composition, antioxidant and antimicrobial activities of free and bound phenolic extracts of Moringa oleifera seed flour. J Funct Foods. 2013; 5(4):1883–91.

49. Roller S. The quest for natural antimicrobials as novel means of food preservation: Status report on a European research project. Int Biodeterior Biodegradation 1995; 36(3-4):333–45.

50. Stratford M, Rose AH. Transport of Sulphur Dioxide by Saccharomyces cerevisiae. Microbiology. 1986; 132(1):1–6.

51. Krebs HA, Wigginst D, Stubbs M. Studies on the mechanism of the antifungal action of benzoate. Biochem J. Sep 15, 1983; 214(3): 657–663.

52. Stratford M, Nebe-von-Caron G, Steels H, Novodvorska M, Ueckert J, Archer DB. Weak-acid preservatives: pH and proton movements in the yeast Saccharomyces cerevisiae. Int J Food Microbiol. 2013; 161(3):164–71.

53. Nanditha B, Prabhasankar P. Antioxidants in bakery products: a review. Crit Rev Food Sci Nutr. 2009; 49(1):1–27.

54. Brewer MS. Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. Compr Rev Food Sci Food Saf. 2011; 10(4):221–47.

55. Nawar W. Lipids. In: Fennema OR, editor. Food chemistry. 3rd ed. New York, NY: Marcel Dekker, Inc; 1996. p. 225–320.

56. Choe E, Min DB. Mechanisms and Factors for Edible Oil Oxidation. Compr Rev Food Sci Food Saf. 2006; 5:169–86.

57. Graf E. Antioxidant functions of phytic acid. Free Radic Biol Med. 1990; 8(1):61–9.

58. DB M, SH L, Lee E. Singlet oxygen oxidation of vegetable oil. In: DB M, Smouse T, editors. Flavor chemistry of lipid foods. Champaign, Ill: AOCS Press; 1989. p. 57–97.

59. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J Nutr Biochem. 2002; 13(10):572–84.

60. Shahidi F, Naczk M. Phenolics in Food and Nutraceuticals. Boca Raton: CRC Press; 2003.

61. Han BINS, Ai YIZC, Un MEIS, Orke HAC. Antioxidant Capacity of 26 Spice Extracts and Characterization of Their Phenolic Constituents. J Agric Food Chem. 2005; 53(20):7749-59.

62. Gheldof N, Ngeseth NIJE. Antioxidant Capacity of Honeys from Various Floral Sources Based on the Determination of Oxygen Radical Absorbance Capacity and Inhibition of in Vitro Lipoprotein Oxidation in Human Serum Samples. J Agric Food Chem. 2002; 50(10):3050-5.

63. Bravo L. Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance. Nutr Rev. 2009; 56(11):317–33.

64. Kozłowska A, Szostak-węgierek D. Flavonoids - Food Sources And Health Benefits. Rocz Panstw Zakl Hig. 2014; 65(2):79-85.

65. Grotewold E, editor. The Science of Flavonoids. New York: Springer New York; 2006.

66. Thilakarathna SH, Rupasinghe HPV. Flavonoid bioavailability and attempts for bioavailability enhancement. Nutrients. 2013; 5(9):3367–87.

67. Rice-evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 1996; 20(7):933–56.

68. Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP, *et al*. Structural Determinants of Phosphoinositide 3-Kinase Inhibition by Wortmannin, LY294002, Quercetin, Myricetin, and Staurosporine. Mol Cell. 2000; 6(4):909–19.

69. Senthilkumar K, Elumalai P, Arunkumar R, Banudevi S, Gunadharini ND, Sharmila G, *et al*. Quercetin regulates insulin like growth factor signaling and induces intrinsic and extrinsic pathway mediated apoptosis in androgen independent prostate cancer cells (PC-3). Mol Cell Biochem. 2010; 344(1-2):173–84.

70. Margina D, Ilie M, Manda G, Neagoe I, Mocanu M, Ionescu D, *et al*. Quercetin and epigallocatechin gallate effects on the cell membranes biophysical properties correlate with their antioxidant potential. Gen Physiol Biophys. 2012; 31(1):47–55.

71. Kucharska AZ, Oszmian J, Bakowska, A. The effects of heating , UV irradiation , and storage on stability of the anthocyanin – polyphenol copigment complex. Food Chem. 2003; 81:349–355.

72. Castañeda-Ovando A, Pacheco-Hernández MDL, Páez-Hernández ME, Rodríguez J a., Galán-Vidal CA. Chemical studies of anthocyanins: A review. Food Chem. 2009; 113(4):859–71.

73. Desai KGH, Jin Park H. Recent Developments in Microencapsulation of Food Ingredients. Drying Technol: An Int J. 2005; 23(7):1361-1394.

74. Shahidi F, Han X. Encapsulation of food ingredients. Crit. Rev. Food Sci. Nutr. 1993; 33(6): 501-547.

75. Kwak H-S, editor. Nano-and Microencapsulation for Foods. West Sussex: John Wiley& Sons, Ltd; 2014.

76. Fang Z, Bhandari B. Encapsulation of polyphenols – a review. Trends Food Sci Technol. 2010; 21(10):510–23.

77. Fu N, Zhou Z, Jones TB, Tan TTY, Wu WD, Lin SX, *et al*. Production of monodisperse epigallocatechin gallate (EGCG) microparticles by spray drying for high antioxidant activity retention. Int J Pharm. 2011; 413(1-2):155–66.

78. Gouin S. Microencapsulation: industrial appraisal of existing technologies and trends. Trends Food Sci Technol. 2004; 15(7-8):330–47.

79. Nakagawa K, Nagao H. Microencapsulation of oil droplets using freezing-induced gelatin–acacia complex coacervation. Colloids Surfaces A Physicochem Eng Asp. 2012; 411:129–39.

80. Zeiger E, Gollapudi B, Spencer P. Genetic toxicity and carcinogenicity studies of glutaraldehyde--a review. Mutat Res. 2005; 589(2):136–51.

81. Gouin S. Micro-encapsulation: industrial appraisal of existing technologies and trends. Trends Food Sci Technol. 2004; 15(7-8):330–47.

82. Santos MG, Bozza FT, Thomazini M, Favaro-Trindade CS. Microencapsulation of xylitol by double emulsion followed by complex coacervation. Food Chem. 2015; 171:32–9.

83. Zuidam NJ, Nedovic V, editors. Encapsulation Technologies for Active Food Ingredients and Food Processing. New York, NY: Springer New York; 2010;

84. Isailović BD, Kostić IT, Zvonar A, Đorđević VB, Gašperlin M, Nedović V a., *et al*. Resveratrol loaded liposomes produced by different techniques. Innov Food Sci Emerg Technol. 2013; 19:181–9.

85. Chen AC, Veiga MF, Rizzuto AB. Cocrystallization: an encapsulation process. Food Technol. 1988; 11:87–90.

86. Beristain CI, Instituto AV, B DC. Encapsulation of Orange Peel Oil by Co-crystallization. 1996; 647:645–7.

87. López-Córdoba A, Deladino L, Agudelo-Mesa L, Martino M. Yerba mate antioxidant powders obtained by co-crystallization: Stability during storage. J Food Eng. 2014; 124:158–65.

88. Oetjen GW, Haseley P. Freeze-drying. Weinheim, Germany: WileyVCH Verlag Gmbh & Co. KGaA; 2004.

89. Pérez-Gregorio MR, Regueiro J, González-Barreiro C, Rial-Otero R, Simal-Gándara J. Changes in antioxidant flavonoids during freeze-drying of red onions and subsequent storage. Food Control. 2011; 22(7):1108–13.

90. Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine. 2006; 2(1):8–21.

91. Donsì F, Annunziata M, Sessa M, Ferrari G. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. LWT - Food Sci Technol. 2011; 44(9):1908–14.

92. F. Gibbs, Selim Kermasha, Inteaz Al B. Encapsulation in the food industry: a review. Int J Food Sci Nutr. Informa UK Ltd UK; 1999; 50(3):213–24.

93. McClements DJ, Decker EA, Park Y, Weiss J. Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. Crit Rev Food Sci Nutr. 2009; 49(6):577-606.

94. Sauvant P, Cansell M, Hadj Sassi A, Atgié C. Vitamin A enrichment: Caution with encapsulation strategies used for food applications. Food Res Int. 2012; 46(2):469–79.

95. McClements DJ, Decker EA, Park Y, Weiss J. Designing Food Structure to Control Stability, Digestion, Release and Absorption of Lipophilic Food Components. Food Biophys. 2008; 3(2):219–28.

96. Szejtli J. Introduction and General Overview of Cyclodextrin Chemistry. Chem Rev 1998; 98 (5):1743–1754.

97. Bilensoy E, editor. Cyclodextrins in Pharmaceutics, Cosmetics, and Biomedicine: Current and Future Industrial Applications. Hoboken, New Jersey: John Wiley & Sons, Inc; 2011.

98. Saenger W, Jacob J, Gessler K, Steiner T, Hoffmann D, Sanbe H, Koizumi K, Smith SM, Takaha T. Structures of the Common Cyclodextrins and Their Larger Analogues-Beyond the Doughnut. Chem Rev. 1998; 98(5):1787-1802.

99. Villiers, A. Sur la fermentation de la fécule par l'action du ferment butyrique. C R Acad Sci 1891; 112: 536-38.

100. Schardinger, F. Über thermophile Bakterien aus verschiedenen Speisen und Milch. Z. Unters. Nahr. Genussm. 1903; 6(19):865-80.

101. Schardinger F. Schardinger dextrins. Zentralbl Bakteriol, Parasitenkd, Infekt Hyg, Abt 2. 1911; 29(188).

102. Crini G. Review: A History of Cyclodextrins. Chem. Rev. 2014; 114 (21):10940–10975.

103. Pringsheim H, Langhans A. Über krystallisierte Polysaccharide aus Stärke. Berichte der Dtsch Chem Gesellschaft. 1912; 45(2):2533–46.

104. Pringsheim H, Eissler F. Über Schardingers krystallisierte Dextrine II. Berichte der Dtsch Chem Gesellschaft. 1913; 46(3):2959–74.

105. Freudenberg K, Blomqvist G, Ewald L, Soff K. Hydrolyse und Acetolyse der Stärke und der Schardinger-Dextrine. Berichte der Dtsch Chem Gesellschaft. 1936;69(6):1258–66.

106. Freudenberg K, Cramer F. Die Konstitution der Schardinger-Dextrine α, β und γ. Zeitschrift für Naturforschung B. 1948; 3(11-12): 464–66.

107. Freudenberg K, Schaaf E, Dumpert G, Ploetz T. Neue Ansichten Über die Stärke. Naturwissenschaften 1939; 27(51):850–3.

108. French D, Rundle RE. The Molecular Weights of the Schardinger Alpha and Beta Dextrins 1. J Am Chem Soc. 1942; 64(7):1651–3.

109. Dietrich H V., Cramer F. Über Einschlußverbindungen, VII. Mitteil: Zur Struktur der Jodketten in Kanal-Einschlußverbindungen. Chem Ber. 1954; 87(6):806–17.

110. Cramer F. Einschlußverbindungen. Angew Chemie. 1956; 68(3):115–20.

111. Frömming K-H, Szejtli J. Cyclodextrins in Pharmacy. Dordrecht: Springer Netherlands; 1993.

112. Szejtli J. Cyclodextrin Technology. Dordrecht: Springer Netherlands; 1988.

113. Szejtli J. Utilization of cyclodextrins in industrial products and processes. J. Mater. Chem. 1997; 7: 575-87.

114. Tomasik P, editor. Chemical and Functional Properties of Food Saccharides. Boca Raton, Florida: CRC Press; 2003.

115. Szejtli J. Past, present and futute of cyclodextrin research. Pure Appl Chem. 2004; 76(10):1825–45.

116. http://www.cyclolab.hu/ [cited 2015 May 22].

117. Starnes RL, Trackman PC, Katkocin DM, inventors; Novo Industri As., assignee. Thermostable cyclodextrin glycosyl transferase, its production and use. WO 1989003421 A1. 1989.

118. Park K-H, Robyt JF, Choi Y-D, editors. Enzymes for Carbohydrate Engineering Amsterdam: Elsevier; 1996.

119. Armbruster FC, Kooi ER, inventors; Corn Products Co, assignee. Production of cyclodextrin. US 3425910 A. 1969.

120. Biwer A, Antranikian G, Heinzle E. Enzymatic production of cyclodextrins. Appl Microbiol Biotechnol 2002; 59(6):609–17.

121. Okada M, Matsuzawa M, Uezima O, Nakakuki T, Horikoshi K, inventors; Nihon Shokuhin Kako Co., Ltd., Rikagaku Kenkyusho, assignees. Process for producing gamma-cyclodextrins. US 4418144 A. 1983.

122. Ibrahim ASS, El-Tayeb MA, Elbadawi YB, Al-Salamah AA. Effects of substrates and reaction conditions on production of cyclodextrins using cyclodextrin glucanotransferase from newly isolated Bacillus agaradhaerens KSU-A11. Electron J Biotechnol 2011; 14(5).

123. Considine GD, editor. Inclusion compounds. In: Van Nostrand's Encyclopedia of Chemistry. Hoboken, New Jersey: John Wiley & Sons, Inc; 2005. 5th edition p. 824-25.

124. Astray G, Gonzalez-Barreiro C, Mejuto JC, Rial-Otero R, Simal-Gándara J. A review on the use of cyclodextrins in foods. Food Hydrocoll 2009; 23(7):1631–40.

125. Liu J, Mendoza S, Román E, Lynn MJ, Xu R and Kaifer AE. J. Am. Chem. Soc. 1999; 121(17):4304–05.

126. Frank DW, Gray JE, Weaver RN. Cyclodextrin Nephrosis in the Rat. Am J Pathol. 1976; 83(2):367–82.

127. CAVAMAX®, CAVITRON® and CAVASOL® Cyclodextrins - Performance-enhancing products for pharmaceuticals [pamphlet]. New Jersey: International Specialty Products; 2007.

128. Dodziuk H, editor. Cyclodextrins and Their Complexes: Chemistry, Analytical Methods, Applications. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2006.

129. Loftsson T, Brewster ME. Pharmaceutical applications of cyclodextrins: basic science and product development. J Pharm Pharmacol 2010; 62(11):1607–21.

130. Knittel D, Schollmeyer E. Technologies for a New Century. Surface Modification of Fibres. J Text Inst 2000; 91(3):151–65.

131. Khanna P, Dworschack R, inventors. Microgenics Corporation, assignee. Cleaning using cyclodextrin. EP 0301847 A1. 1989.

132. Ghoul Y El, Martel B, Achari A El, Campagne C, Razafimahefa L, Vroman I. Improved dyeability of polypropylene fabrics finished with β-cyclodextrin–citric acid polymer. Polym J. 2010; 42(10):804–11.

133. Wacker Chemie AG. WETSOFT® - Global. Discover new horizons – Wacker textile finishing [pamphlet]. München: Wacker Chemie AG; 2014

134. Szejtli, J. Cyclodextrins in the Textile Industry. Starch – Stärke 2003; 55(5): 191–96.

135. Szente L, Szemán J. Cyclodextrins in analytical chemistry: host-guest type molecular recognition. Anal Chem. 2013; 85(17):8024–30.

136. Moon J-Y, Jung H-J, Moon MH, Chung BC, Choi MH. Inclusion complex-based solid-phase extraction of steroidal compounds with entrapped β-cyclodextrin polymer. Steroids. 2008; 73(11):1090–7.

137. Vasquez JM, Vu A, Schultz JS, Vullev VI. Fluorescence Enhancement of Warfarin Induced by Interaction with β-Cyclodextrin. Biotechnol Prog. 2009; 25(4):906-14.

138. Huber O, Szejtli J, editors. Proceedings of the Fourth International Symposium on Cyclodextrins. Dordrecht: Springer Netherlands; 1988.

139. U.S. Food and Drug Administration. GRAS notice for Alpha-Cyclodextrin. 2004 June 25. GRN No 155.

140. U.S. Food and Drug Administration. GRAS notice for Gamma-Cyclodextrin. 2000 May 22. GRN No 46.

141. U.S. Food and Drug Administration. GRAS notice for Beta-Cyclodextrin. 2001 Mar 23. GRN No 74.

142. European Parliament. Commission implementing decision of 1 June 2012 authorising the placing on the market of Gamma-Cyclodextrin as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council. Official Journal of the European Union. Luxembourg: Publications Office of the European Union; 2012.

143. European Parliament. Commission regulation (EU) No 536/2013 of 11 June 2013 amending Regulation (EU) No 432/2012 establishing a list of permitted health claims made on foods other than those referring to the reduction of disease risk and to children's development and health. Official Journal of the European Union. Luxembourg: Publications Office of the European Union; 2013.

144. European Parliament. Commission regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to

Regulation (EC) No 1333/2008 of the European Parliament and of the Council. Official Journal of the European Union. Luxembourg: Publications Office of the European Union; 2012.

145. Pinho E, Grootveld M, Soares G, Henriques M. Cyclodextrins as encapsulation agents for plant bioactive compounds. Carbohydr Polym. 2014; 101:121–35.

146. Kant A, Linforth RST, Jort J, and Taylor AJ. Effect of β-Cyclodextrin on Aroma Release and Flavor Perception. J. Agric. Food Chem. 2004; 52:2028−35.

147. Tamamoto LC, Schmidt SJ, Lee S-Y. Sensory properties of ginseng solutions modified by masking agents. J Food Sci. 2010; 75(7):S341–7.

148. Wang J, Cao Y, Sun B, Wang C. Physicochemical and release characterisation of garlic oil-β-cyclodextrin inclusion complexes. Food Chem 2011; 127(4):1680–5.

149. Bhandari BR, D'Arcy BR and Bich LLT. Lemon Oil to β-Cyclodextrin Ratio Effect on the Inclusion Efficiency of β-Cyclodextrin and the Retention of Oil Volatiles in the Complex. J. Agric. Food Chem. 1998; 46:1494−99.

150. Wacker Chemie AG. Info sheet I CAVAMAX® W6. Stabilizing oil-in-water emulsions with ALPHADEXTRIN [pamphlet]. München: Wacker Chemie AG; 2013.

151. Kwak HS, Kim SH, Kim JH, Choi HJ, Kang J. Immobilized β-Cyclodextrin as a Simple and Recyclable Method for Cholesterol Removal in Milk. Arch Pharm Res 2004; 27(8):873-77.

152. Kim JJ, Jung TH, Ahn J, Kwak HS. Properties of cholesterol-reduced butter made with β-cyclodextrin and added evening primrose oil and phytosterols. J Dairy Sci 2006; 89(12):4503–10.

153. Kwak HS, Nam CG, Ahn J. Low Cholesterol Mozzarella Cheese Obtained from Homogenized and β-Cyclodextrin-Treated Milk. Asian Australas. J. Anim. Sci 2001; 14(2): 268-75.

154. Shim SY, Ahn J, Kwak HS. Functional properties of cholesterol-removed whipping cream treated by β -Cyclodextrin. J Dairy Sci 2003; 86(9):2767–72.

155. López-Nicolas JM, García-Carmona F. Use of cyclodextrins as secondary antioxidants to improve the color of fresh pear juice. J Agric Food Chem. 2007; 55(15):6330-338.

156. López-Nicolas JM, Pérez-López AJ, Carbonell-Barrachina A, García-Carmona F. Use of natural and modified cyclodextrins as inhibiting agents of peach juice enzymatic browning. J Agric Food Chem. 2007; 55(13):5312-319.

157. Hicks KB, Sapers GM, Seib PA, inventors. The United States Of America As Represented By The Secretary Of Agriculture, assignee. Process for preserving raw

fruit and vegetable juices using cyclodextrins and compositions thereof. US 4975293 A. 1990.

158. Szente L, Szejtli J. Cyclodextrins as food ingredients. Trends Food Sci Technol. 2004; 15(3-4):137–42.

159. López-de-Dicastillo C1, Jordá M, Catalá R, Gavara R, Hernández-Muñoz P. Development of Active Polyvinyl Alcohol/β-Cyclodextrin Composites To Scavenge Undesirable Food Components. J. Agric. Food Chem. 2011; 59:11026–033.

160. Grigoriu A, Luca C, Horoba E, Dunca S. Antimicrobial Polymer Films Functionalized with Cyclodextrins. Rev. Chim. (Bucharest) 2013; 64 (6): 606-11.

161. Bonar, A. Functional foods market is expected to grow 25% by 2017: Leatherhead [Internet]. 2014 [updated 2014 Nov 25; cited 2015 Jan 28]. Available from: http://www.nutraingredients.com/Markets-and-Trends/Functional-foods-market-is-expected-to-grow-25-by-2017-Leatherhead?utm_source=copyright&utm_medium=OnSite&utm_campaign=copyright

162. Santos IS, Ponte BM, Boonme P, Silva AM, Souto EB. Nanoencapsulation of polyphenols for protective effect against colon-rectal cancer. Biotechnol Adv. 2013; 31(5):514–23.

163. Nday CM, Halevas E, Jackson GE, Salifoglou A. Quercetin encapsulation in modified silica nanoparticles: potential use against Cu(II)-induced oxidative stress in neurodegeneration. J Inorg Biochem. 2015; 145:51–64.

164. Cai X, Fang Z, Dou J, Yu a., Zhai G. Bioavailability of Quercetin: Problems and Promises. Curr Med Chem. 2013; 20(20):2572–82.

165. Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents. 2005; 26(5):343–56.

166. Ravichandran R, Rajendran M, Devapiriam D. Antioxidant study of quercetin and their metal complex and determination of stability constant by spectrophotometry method. Food Chem. 2014; 146:472–8.

167. Viscidi K a., Dougherty MP, Briggs J, Camire ME. Complex phenolic compounds reduce lipid oxidation in extruded oat cereals. LWT - Food Sci Technol. 2004; 37(7):789–96.

168. Zhao M, Wang H-L, Zhang L, Zhao C, Ji L-N, Mao Z-W. Unexpected phosphodiesterase activity at low pH of a dinuclear copper-β-cyclodextrin complex. Chem Commun (Camb) 2011; 47(26):7344–346.

169. Lachman L, Lieberman HA, Kanig JL. The Theory and Practice of Industrial Pharmacy. 3rd edition. New Delhi: CBS Publisher & Distributors P Ltd 2010.

2. Experimental Section

2. Experimental Section

2.1 Preparation and Characterization of the Compounds

2.1.1 Materials

Table 6 – Description of the materials used in the work.

Reagent	***Supplier***
Preparation of the Complexes	
β-Cyclodextrin	Wacker Chemie AG (Munich, Germany)
γ-Cyclodextrin	
Quercetin	Discovery Fine Chemicals (United Kingdom)
Acetone (≥99.5%)	VWR, BDH Prolabo (Carnaxide, Portugal)
Ethanol (≥99.5%)	Riedel-de Haën (Seelze, Germany)
Thiobarbituric Acid Reactive Substances Assay (TBARS)	
2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH)	Sigma – Aldrich (Sintra, Portugal)
Thiobarbituric Acid	
Ethanol (>99.5%)	PanReac AppliChem (Darmstadt, Germany)
Trichloroacetic Acid	Farbwerke Hoechst AG (Frankfurt, Germany)
Radical Scavenging Assay	
2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$)	Sigma – Aldrich (Sintra, Portugal)
Methanol (≥99.5%)	
Ethanol (>99.5%)	PanReac AppliChem (Darmstadt, Germany)

A quality control of quercetin by vibrational spectroscopy (FTIR) was performed to confirm the quality and purity of this compound, with positive results.

2.1.2 Preparation of the Compounds

2.1.2.1 Attempted Preparation of the Compounds by the Co-Crystalization Method

β-CD·Quercetin with 1:1 Stoichiometry

200mg (0.152 mmol) of β-CD were dissolved in a solvent mixture of water and ethanol (80/20 $^{v}/_{v}$) heated at 60°C. After dissolution of the cyclodextrin, 46mg (0.152 mmol) of quercetin were carefully added, paying attention to its dissolution. In the case of quercetin not dissolving, small volumes of ethanol were added, not exceeding the proportion 50/50 ($^{v}/_{v}$) water/ethanol. The resulting solution was kept in agitation for 3 hours and a half and then transferred to a crystallization (for slow cooling), being left there for roughly two days. Whenever necessary, the mixture was further stored in the fridge to maximize the formation of crystals.

γ-CD·Quercetin with 1:1 Stoichiometry

200mg (0.14 mmol) of γ-CD were dissolved in 5mL of water and put in a water bath at 60°C. 42mg (0.14 mmol) of solid quercetin were then added, but the guest did not dissolve in the aqueous environment, requiring the addition of 5mL of ethanol for dissolution. The resulting clear yellow solution was stirred for 4h further, and then transferred to a crystallization dewar container for roughly two days. Whenever necessary, the mixture was further stored in the fridge to maximize the formation of crystals.

2.1.2.2 Preparation of the Compounds by the Freeze-Drying Method

β-CD·Quercetin with 1:1 Stoichiometry

100mg (0.076 mmol) of β-CD were dissolved in 10mL of water and put under agitation at 37°C. Separately, 23mg (0.076 mmol) of quercetin were dissolved in 13mL of ethanol and also stirred at 37°C. After full dissolution of both compounds, their solutions were mixed, and the resulting solution was stirred for 3 hours further. After this, the resulting solution was snap-frozen by liquid nitrogen and placed in a 1.5 liters MicroModulyo Freeze Dryer from Thermo Electron for c.a. 3 days. The resulting pale yellow

voluminous solid was separated into two fractions, one being kept in a desiccator (β-CD·Quercetin with 1:1 stoichiometry) and the other placed in beaker inside a closed vessel with a water-saturated atmosphere (β-CD·Quercetin with 1:1 stoichiometry rehydrated).

γ-CD·Quercetin with 1:1 Stoichiometry

Two methods were used for the preparation of the compound γ-CD·Quercetin with 1:1 stoichiometry, with equivalent results. First, 200mg (0.14 mmol) of γ-CD were dissolved in 7mL of water and then 42mg (0.14 mmol) of quercetin were carefully added to this solution. Because quercetin would not dissolve, 20mL of ethanol were added. After full dissolution of both compounds, the resulting solution was snap-frozen with liquid nitrogen and freeze-dried. The second method consisted in the dissolution of 100mg of γ-CD in 14mL of water and 21mg of quercetin in 11mL of acetone, in separate solutions. After full dissolution of both compounds, these two solutions were mixed and kept in agitation at room temperature for 2 hours. The final solution was then snap-frozen with liquid nitrogen and freeze-dried for c.a. 3 days.

2.1.3 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra were obtained using KBr pellets, averaging 64 scans at a resolution of 2 cm^{-1}, in a Mattson instruments inc. 7000 galaxy series FT-IR 7000. The main absorption bands observed are presented in Table 7.

Table 7 - Absorption peaks in FTIR spectroscopy of the guest molecule and the prepared compounds. The intensity of the bands follows the code: vs - very strong, s - strong, m - medium, w - weak and vw - very weak.

Compound	Peaks(ν_{max}/cm^{-1})
Quercetin	3408 vs; 3322 vs; 1664 m; 1611 vs; 1560 m; 1523 vs; 1459 m; 1450 m; 1408 m; 1383 s; 1319 s; 1262 vs; 1216 s; 1200 s; 1169 s; 1132 m; 1092 w; 1014 m; 942 w; 865 w; 843 w; 825 s; 796 m; 785 w; 722 m; 703 w; 681 m; 658 w, 639 m; 604 w.
β-CD·Quercetin	3400 vs; 2924 m; 2362 w; 1664 w; 1612 m; 1561 m; 1523 m; 1450 w; 1408 w; 1383 m; 1369 w; 1322 m; 1263 m; 1200 w; 1169 s; 1103 m; 1081 s; 1029 vs; 949 w; 940 w; 865 w; 843 w; 825 w; 795 w; 755 m; 705 m; 668 m; 606 m; 576 m.
γ-CD·Quercetin	3404 vs; 2926 m; 1658 m; 1629 m; 1613 m; 1566 w; 1523 m; 1460 w; 1449 w; 1410 w; 1384 w; 1322 w; 1264 w; 1200 w; 1159 s; 1105 m; 1079 s; 1027 vs; 942 w; 865 w; 842 w; 760 w; 638 w; 604 w; 581 w.

2.1.4 Raman Spectroscopy

Raman spectra were collected in a Bruker RFS 100/S spectrometer equipped with an Nd:YAG laser (1064 nm), working typically between 30-50 mW at the sample position. The main bands observed are presented in Table 8.

Table 8 - Intensity peaks in Raman spectroscopy of the guest molecule and the prepared compounds. The intensity of the bands follows the code: vs - very strong, s - strong, m - medium, w - weak and vw - very weak.

Compound	***Peaks***
Quercetin	1660 w; 1605 vs; 1589 m; 1546 s; 1439 s; 1400 m; 1370 m; 1327 m; 1317 m; 1293 w; 1267 vw; 1219 w; 1175 w; 1137 vw; 1112 w; 1093 vw; 1012 vw; 993 vw; 942 m; 842 m; 784 s; 721 w; 706 w; 684 w; 660 w; 638 m; 603 s; 577 m.
β-CD:Quercetin	1660 w; 1605 vs; 1589 m; 1547 s; 1439 s; 1399 m; 1371 m; 1327 m; 1316 m; 1294 w; 1267 w; 1219 w; 1174 w; 1137 vw; 1112 w; 1093 vw; 1014 vw; 994 vw; 942 m; 843 m; 784 m; 721 w; 706 w; 685 w; 660 w; 639 m; 603 s.
γ-CD:Quercetin	1564 w; 1606 vs; 1590 m; 1567 m; 1548 m; 1441 s; 1401 s; 1371 m; 1318 s; 1267 w; 1217 w; 1175 w; 1112 m; 1084 w; 995 w; 942 w; 843 w; 784 w; 721 w; 707 w; 687 w; 659 w; 638 m; 602 s.

2.1.5 Thermogravimetric Analysis (TGA)

TGA studies were performed on a Shimadzu TGA-50 thermogravimetric analyser with a heating rate of 5°C.min^{-1}, operating under air, with a flow of 20 mL.min^{-1}. The sample holder was a platinum plate with 6 mm ∅ and the sample mass was typically between 5 and 10 mg. The thermogravimetric characteristics of the guest and host molecules and compounds prepared are summarized in Table 9.

Table 9 - Thermogravimetric characteristics of the guest and host molecules and prepared compounds.

	Mass Loss Process		
Compound	***1***	***2***	***3***
Quercetin			
Temperature range	86-115°C	275-355°C	355-510°C
% Mass loss	10%	19%	64%
β-CD			
Temperature range	20-75°C	240-350°C	350-519°C
% Mass loss	15%	53%	34%
β-CD·Quercetin			
Temperature range	20-80°C	270-308°C	308-491°C
% Mass loss	11%	39%	47%
γ-CD			
Temperature range	20-87°C	255-328°C	330-519°C
% Mass loss	15%	65%	20%
γ-CD·Quercetin			
Temperature range	20-90°C	250-334°C	330-519°C
% Mass loss	15%	41%	20%

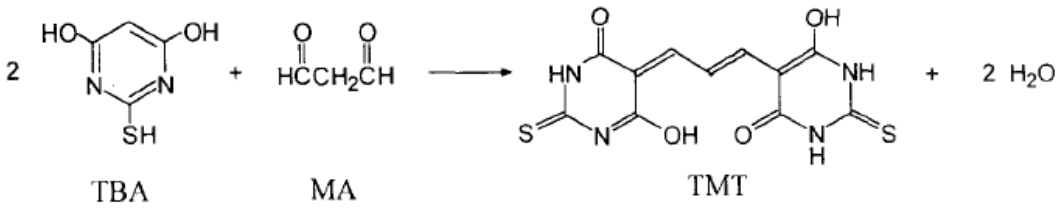
2.1.6 Powder X-Ray Diffraction (PXRD)

The powder x-ray diffraction data (DXRP) was collected at room temperature in a PANalytical Empyrean diffractometer with a copper radiation source (Cu-Kα_1, λ = 1.5406 Å), with a rotatory plate support in a Bragg-Brentano configuration (45 kV and 40 mA). The intensity data was collected through the step counting method (step 0.0001) in the 3.5°≤ 2θ ≤ 50.0° interval.

2.2 Antioxidant Capacity Assays

2.2.1 Anti-Peroxidation Assay – TBARS

The anti-peroxidation test currently performed was an adaptation of the thiobarbituric acid reactive substances assay (TBARS) (1), which relies on the reaction between malonaldehyde (MA) and thiobarbituric acid (TBA) (Scheme 4) to produce a compound which can be quantified spectrophotometrically at 532 nm (2).



Scheme 4 – Formation of the product spectroscopically detectable from the reaction between thiobarbituric acid (TBA) and malonaldehyde (MA) (2).

Briefly, a radical source is added to the compound under study at various concentrations, followed by incubation for a certain period of time for the reaction to occur. A peroxidation inducer might be added in this step, to reduce the time of the experience. Then, the peroxidation reaction is stopped using an acid solution, and thiobarbituric acid is added as an indicator for the peroxidation products generated. Afterwards, the absorbance of this solution is measured (at 532 nm), allowing to quantify of the anti-peroxidation capacity of the compound.

The reagents used in this assay were an egg yolk buffered solution (phosphate buffer at 0.1M) as a radical source, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 0.12M as an additional radical source to speed up the reaction and enhance the detection of radicals, trichloroacetic acid at 15% ($^{v}/_{v}$), as the acidic solution that stops the peroxidation reactions, and thiobarbituric acid at 1% ($^{v}/_{v}$). The sample solutions were prepared dissolving the cyclodextrin-inclusion (CD-inclusion) compounds in ethanol at 70% ($^{v}/_{v}$) and quercetin in ethanol at 96% ($^{v}/_{v}$), in concentrations ranging from 0.1 mM to 1 mM. The absorbance of the sample solutions was measured in a µQuant™ Microplate Spectrophotometer from BioTek Instruments, Inc, at 532 nm.

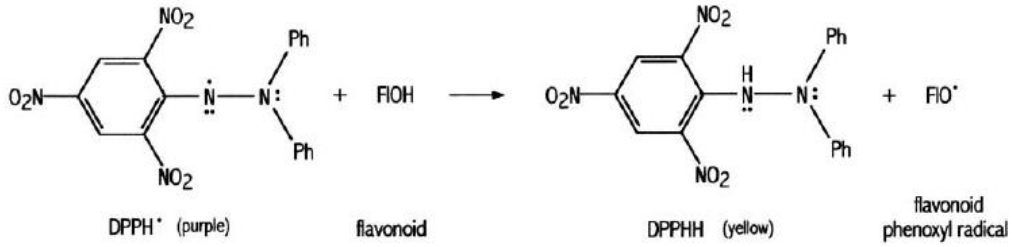
The percentage of inhibition of peroxidation was calculated using the formula:

$$[1] \qquad \% \text{ of inhibition} = \frac{A_0 - A_s}{A_0} \times 100$$

where A_0 and A_s represent the absorbance of the control and of the sample, respectively. The control is composed by the same reagents, without the CD-inclusion compounds (the same volume of solvent is added to the egg yolk solution).

2.2.2 Radical Scavenging Assay – DPPH

The DPPH assay is a test commonly used for assessing the antioxidant capacity of flavonoids. It relies on the measurement of the sample's ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radicals (which are purple), affording a yellow product (Scheme 5).



Scheme 5 - Radical scavenging reaction between a flavonoid and DDPH (3).

This process is monitored by measuring the absorbance around 517 nm, at prescribed intervals, in a multi-well reader (Figure 6). The assay is considered as finished when no more changes in absorbance are found between two consecutive measurements (3).

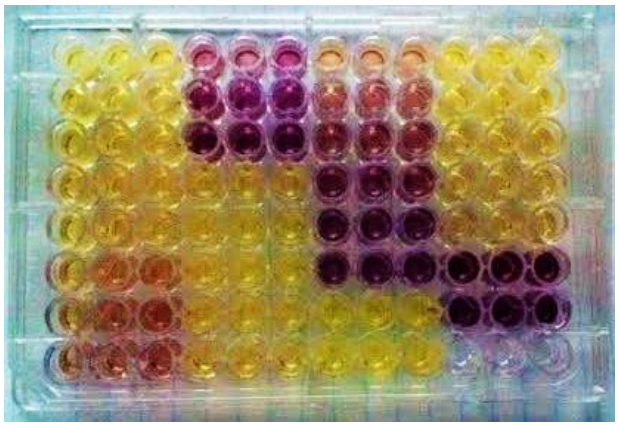


Fig. 6 – Multi-well plate containing the solutions tested by the DPPH assay.

For this assay, a methanolic solution of DPPH (0.5mM) was added to various sample solutions, in concentrations ranging from 0.01 to 0.2 mM, for the CD-inclusion compounds, and 0.0005 to 1 mM, for pure quercetin. The solvents for the sample solutions were ethanol at 70% ($^{v}/_{v}$) for the CD-inclusion compounds and ethanol at 96% ($^{v}/_{v}$) for quercetin. The absorbance of the various solutions was measured at 515nm, in the Microplate Spectrophotometer mentioned before.

The measurement of the antioxidant potential of the samples through the DPPH assay is evaluated by the antiradical efficiency (AE), for this method relies in the capacity of radical scavenging by the compound under study. This parameter is calculated using the formula

$$AE = \frac{1}{IC_{50} \times t_{IC50}} \quad [2]$$

where IC_{50} is the concentration necessary to reduce 50% of the DPPH radical in the solution and t_{IC50} is the time that takes to attain this reduction.

2.3 Sensory Analysis

A sensory analysis was performed to evaluate the effect of adding the CD-inclusion compounds to the food matrix. A product preference test was conducted, which evaluates the preference of a given product by a consumer. A panel of 30 non-trained tasters tasted a sample of a normal, non altered, fresh cheese and a sample of a modified fresh cheese, stating their preference and giving some commentary about the products. Treatment of the results was made applying the Pearson's chi-squared test for independence, that allows to determine whether there is a significant difference between two or more variables.

The obtained observations are presented in Table 10.

Table 10- Results from the product preference test carried out for fresh cheese containing CD-inclusion compounds.

Type of compound	Not modified	Added compound	No preference	Total observations[1]
β-CD·Quercetin	14	15	1	29
γ-CD·Quercetin	10	15	5	25

The "no preference" observations were not considered for the total number of observations, for they do not help to access the significant difference between the products.

To apply the Pearson's chi-squared test for independence to these observations the following equation was considered:

[3] $$X^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

Where:

X^2 is the chi squared value for the test; O_i is the number of observations of the variable *i*; E_i is the estimated number for the variable *i*.

The estimated number of the variables can be calculated applying the formula (RowTotal*ColumnTotal)/GridTotal to the results in Table 10. After determination of the X^2 value, it is possible to obtain the *p*-value for the test, which if lower than 0.05, the variables are considered significantly different. For one degree of freedom, the *p*-value for the tested cheeses with β-CD·Quercetin was 0.7, while for the cheeses with γ-CD·Quercetin it was 0.4, which means that there is no significant differences between the not modified cheeses and those with incorporated CD-inclusion compounds.

References

1. Daker M, Abdullah N, Vikineswary S, Goh P, Kuppusamy U. Antioxidant from maize and maize fermented by Marasmiellus sp. as stabiliser of lipid-rich foods. Food Chem. 2007; (107):1092–8.

2. Castrejn SE, Yatsimirsky AK, La S. Cyclodextrin enhanced fluorimetric determination of malonaldehyde by the thiobarbituric acid method. Talanta. 1997; (44):951–7.

3. Amic D, Davidovic-Amic D, Beslo D and Trinajstic N. Structure-Radical Scavenging Activity Relationships of Flavonoids. Croat Chem Acta. 2003; 76(1):55–61.

3. Results and Discussion

3.1 Characterization of the Inclusion Compounds

3.1.1 Vibrational Spectroscopy

3.1.1.1 Fourier Transform Infrared Spectroscopy (FT-IR)

Inclusion of quercetin into the hosts β-CD and γ-CD was investigated by FT-IR, the spectra revealing the presence of the most intense bands of the hosts and of quercetin. Furthermore, a few absorption bands of quercetin exhibit small shifts upon complexation.

The attribution of the quercetin infrared absorption bands was systematically described by Cornard *et al*(1), thus allowing to identify which guest oscillators have suffered changes in frequency upon complexation with the cyclodextrins. Figure 7 presents the atom numbering used in these descriptions.

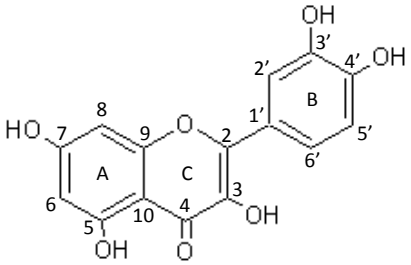


Fig. 7 -Atom numbering for quercetin used in the text.

β-CD·Quercetin

This compounds exhibits only a few small shifts in some of the bands of the guest; these are listed in the Table 11; The 1700 – 850 cm^{-1} region of the spectra collected for quercetin and β-CD·Quercetin are presented in the Figure 8.

Table 11-Assignment of the absorption bands frequencies to the correspondent bonds – Comparison of quercetin with β-CD·Quercetin. (A) and (B) are relative to the benzenic rings and ν=stretching.

Quercetin	*β-CD·Quercetin*	*Assignment*
1611	1612	ν(C2=C3)
1319	1322	ν(C4′-OH)
1262	1263	ν(C-C) of ring B, ν(O1-C2), ν(C3-O3), ν(C3-C4), ν(C4-C10)

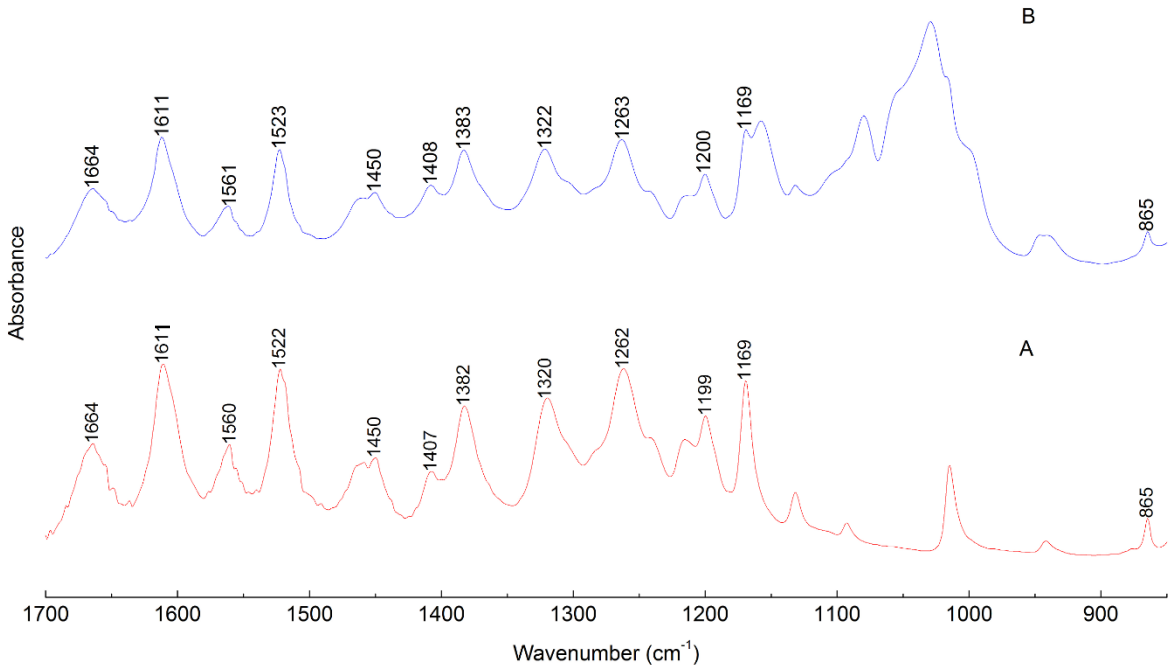


Fig. 8 – FTIR spectra of Quercetin (A) and β-CD·Quercetin complex (B).

These results evidence only a significant shift (3 cm^{-1}) for the C4'-OH oscillator, thus suggesting some kind of host-guest interaction occurring at the catechol moiety (B-ring) of the guest. The other bands present a shift smaller than the equipment resolution and may therefore not be significant. It cannot, thus, be excluded the presence of pure quercetin as a contaminant, as observed by powder X-ray diffraction (discussion of these results are given in detail in the subsections below).

γ-CD·Quercetin

This compound exhibits several bands, associated with guest oscillators, which have suffered shifts upon inclusion into γ-CD; these are listed in the Table 12; The 1700 – 850 cm^{-1} region of the spectra collected for quercetin and γ-CD·Quercetin are presented in the Figure 9.

Table 12-Assignment of the absorption peak frequencies to the correspondent bonds – Comparison of quercetin with the γ-CD·Quercetin complex. (A) and (B) are relative to the benzenic rings and ν=stretching.

Quercetin	*γ-CD·Quercetin*	*Assignment*
1664	1658	ν(C=O)
1560	1566	*ν*(C-C) of ring B
1408	1410	*ν*(C9-O1), *ν*(C2-C1'), *ν*(C5-OH), *ν*(C-C) of ring B
1319	1322	ν(C4'-OH)
1169	1159	*ν*(C-C) of ring B
1262	1264	*ν*(C-C) of ring B, *ν*(O1-C2), *ν*(C3-O3),*ν*(C3-C4), *ν*(C4-C10)

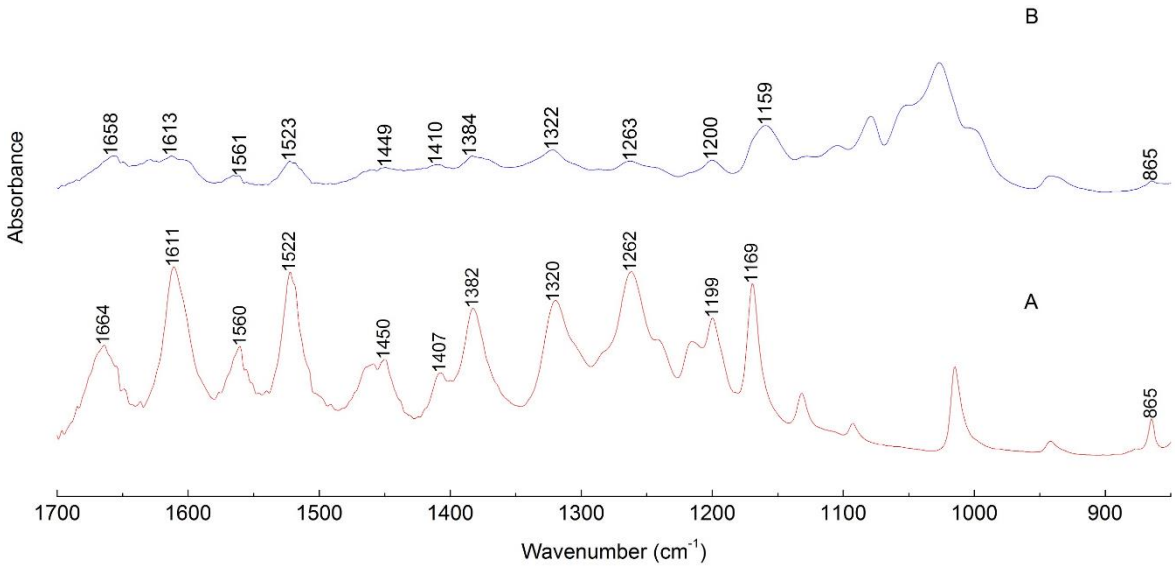
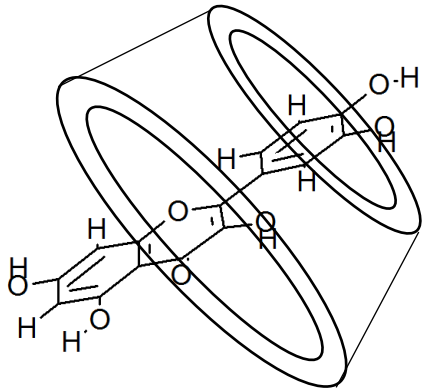


Fig. 9 – FTIR spectra of Quercetin (A) and γ-CD·Quercetin (B).

In γ-CD·Quercetin, the majority of the shifts belong to oscillators in ring B, with some small shifts in oscillators of ring C (O1-C2) and ring A (C5-OH) also being observable. There is a shift in the carbonyl group of ring C, which suggests that the larger cavity of γ-CD allows a deep inclusion involving of this moiety of the guest. A schematic of the possible geometry

of inclusion shows an inclusion of quercetin involving both the catechol and the C ring, as Scheme 6 illustrates.



Scheme 6 - Inclusion of quercetin in γ-CD.

3.1.1.2 Raman Spectroscopy

As with FTIR, the absorption bands which have registered shifts upon inclusion were selected and further analysed. Cornard *et al* also made attributions for Raman vibrations, and their work was once again used as a reference.

β-CD·Quercetin

Regarding the β-CD·Quercetin compound, the Raman spectra obtained didn't present remarkable shifts upon interaction. Considering the results from FTIR, this result is not unexpected, and it is also not abnormal - given that Raman spectroscopy captures vibrations that FTIR doesn't and vice-versa, it is possible that shifts in bond vibration frequency exist even though they are not detectable by a certain technique, which evidences the importance of using various techniques in order to obtain the maximum of information possible about the system in study.

γ-CD·Quercetin

For the γ-CD·Quercetin compound, Raman spectroscopy allowed to obtain more information about the changes in the bond vibrations upon interaction, namely regarding bending modes in ring B. Table 13 presents the intensity bands of quercetin that

demonstrated shifts in their vibration frequency when included into γ-CD. Figure 10 represents the Raman spectra obtained.

Table 13 - Assignment of the Raman intensity peak frequencies to the correspondent bonds – Comparison of quercetin with the γ-CD·Quercetin compound. (A) and (B) are relative to the benzenic rings, ν=stretching and δ = skeleton in-plane bending.

Quercetin	***γ-CD·Quercetin***	***Assignment***
1660	1654	ν(C=O)
1546	1548	ν (C-C) of ring B
1439	1441	ν(C7-OH), ν(C-C) of ring A
1219	1217	ν(C-C) of ring A, δ(C2'-C3'-C4'), δ(C5'-C6'-C1')

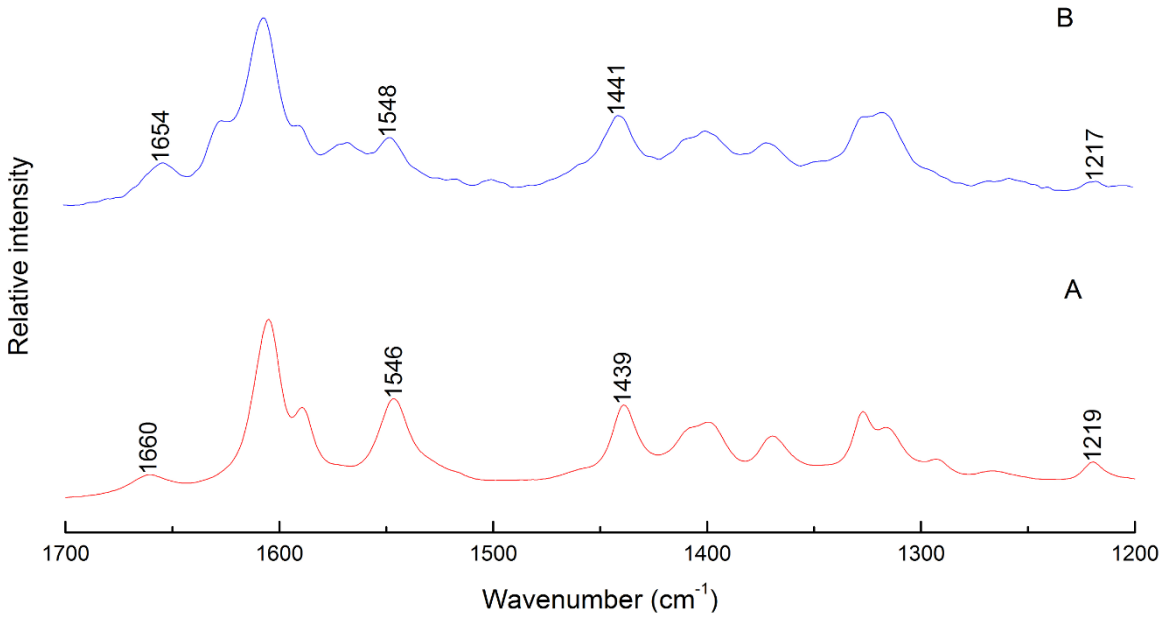


Fig. 10 - Raman spectra of the guest molecule and CD-inclusion compound. A - Quercetin; B - γ-CD·Quercetin. Only the bands that suffered shifts were labelled.

As in FTIR spectroscopy, the bond vibration frequencies shift to higher values, which demonstrates a lowering in the energy of vibration. An hydrogen bond between one of the hydroxyls present in the cyclodextrin cavity and the hydroxyl group in C7 in the γ-CD·Quercetin product might be possible, given that the cavity of the cyclodextrin is wide enough to allow a deeper inclusion of the guest molecule and a shift in the stretching of the C7-OH bond occurs. Raman spectroscopy also confirms that quercetin is more deeply included in γ-CD, through the detection of a shift in the carbonyl bond of ring C.

The obtained vibrational data strongly supports the formation of a true γ CD·Quercetin inclusion complex, such that it changed the electron density around selected bonds with the consequential shift in the bond frequency of vibration. From these results, it was also possible to get some information about the orientation of the guest molecule inside the γ-CD host molecule and thus to postulate on the geometry of complexation for γ-CD·Quercetin.

In the case of β-CD·Quercetin, however, more techniques are needed to help understand the system.

3.1.2. Thermogravimetric Analysis (TGA)

A thermogravimetric analysis of the guest and host molecules and the prepared CD-inclusion compounds was also performed. The TGA curves obtained showed that all these compounds had weight losses in roughly three stages, and allowed to understand the thermal behaviour of the compounds in study.

Quercetin

The thermoanalytical profile for quercetin can be divided into three steps. Initially, there is a 10% mass loss, which corresponds to the loss of water molecules. At approximately 275°C and until 355°C, there is a 19% mass loss related with a partial degradation of quercetin, occurring at temperature slightly above the melting point via an endothermic process (2); the final mass loss is due to ignition, starting at 355°C and proceeding until c.a 510ºC.

β-CD·Quercetin

The thermal behaviour of β-CD and its hydrates has already been well documented and investigated (3), presenting essentially 3 main steps. Initially, there is a slope from 20 to 75°C with a mass loss of 15%, which corresponds to the loss of 11 water molecules. Following this, there is no change in mass until 240°C, and from this temperature until approximately 350°C, a mass loss of 53% related to thermal degradation, together with

oxidation, occurs. From 350°C and above, ignition processes take place, and the molecule registers its last mass loss.

The main differences in the thermal profile of the product β-CD·Quercetin when compared to those of β-CD hydrate and the 1:1 physical mixture of β-CD and quercetin lie in the dehydration step. The mixture clearly shows two dehydration steps, ascribed to waters of hydration of firstly β-CD and then of quercetin, whereas in β-CD·Quercetin the loss of water molecules occurs in a more continuous fashion, with a first mass loss of 11% from room temperature until c.a. 80°C, followed a smaller and smoother mass loss of 4% up to 105ºC. These correspond to the less tightly bound and more tightly bound waters of hydration, respectively. There is no further mass loss until 270°, temperature which marks the onset of thermal degradation for the host in both the mixture (Mx β-CD·Quercetin) and β-CD·Quercetin, the later presenting a bigger mass loss. Considering that quercetin starts its degradation at around 300°C, it's possible that it contributes to the step in the 300-350°C window of the physical mixture (4). Figure 11 shows the thermoanalytical profiles for the host β-CD.

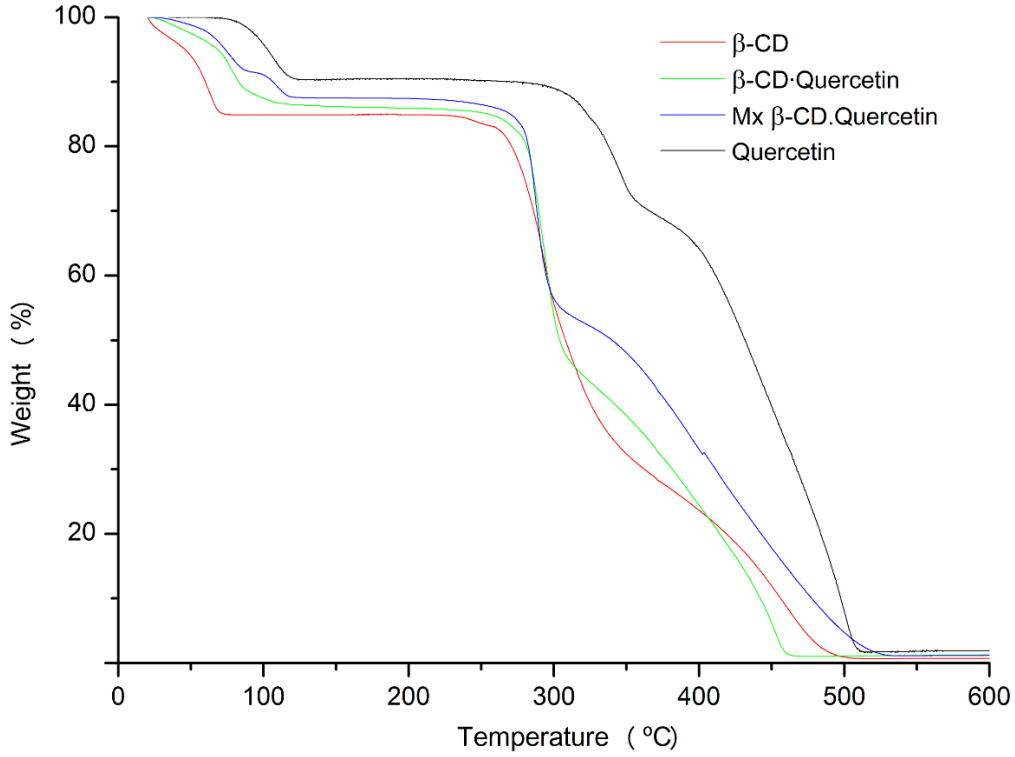


Fig. 11 -TGA profile for the host β-CD.

γ-CD·Quercetin

The thermal profile of γ-CD is similar to β-CD, with the main differences residing in the steepness of the curves representing the various thermal processes. There is a 15% mass loss until 87°C, due to the loss of 14 water molecules, and from 255°C to 328°C a mass loss of 65% occurs, related with thermal degradation and oxidation. Ignition starts right after this, causing the final mass loss.

The TGA curve for the γ-CD·Quercetin product is similar to the one of γ-CD, with a major difference being noticeable in the mass loss in the interval 255-334°C. With pure cyclodextrin, the mass loss is of 65%, while the complex registers a mass loss of 41%. It is also possible to observe a thermic protection of quercetin by γ-CD, for the first degradation of the flavonol is not observed in the complex with γ-CD. Figure 12 shows the thermoanalytical profiles for the host γ-CD.

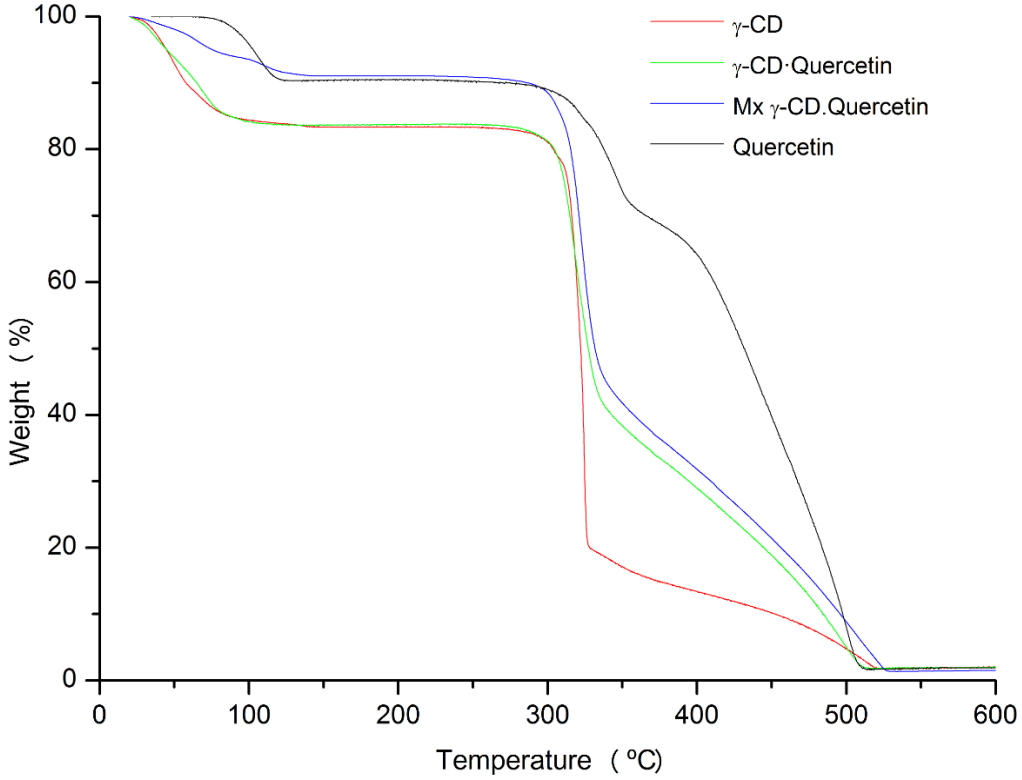


Fig. 12-TGA profile for the host γ-CD.

The degradation step for γ-CD·Quercetin is similar to the one of the physical mixture Mx γ-CD·Quercetin. This result, besides helping to confirm the stoichiometry, also reveals

that there is a good affinity between these two components, which can also be confirmed by the absence of the small heap related with the degradation of pure quercetin.

Overall, it is possible to point some alterations generated by the interaction with cyclodextrins. The water loss processes are altered, with the CD-inclusion compounds having a more steady and gradual loss of water molecules. In the γ-CD·Quercetin compound, as mentioned, there's a thermic protection of the guest molecule by the host, which is an important point when considering the incorporation of such complexes in foods that require heat treatment.

3.1.3 Powder X-Ray Diffraction

Powder X-ray diffraction is a useful method for the detection of cyclodextrin complexation in powder or microcrystalline states (5). If there is no inclusion, the experimental PXRD trace will be the resultant of the superimposition of the patterns of the separated compounds that compose the complex (6), so the diffraction pattern of the complex should be clearly distinct from that of the superimposition of each of the components if a true inclusion compound has been formed (7).

β-CD·Quercetin

The diffractograms for quercetin, β-cyclodextrin and β-CD·Quercetin are presented in Figure 13.

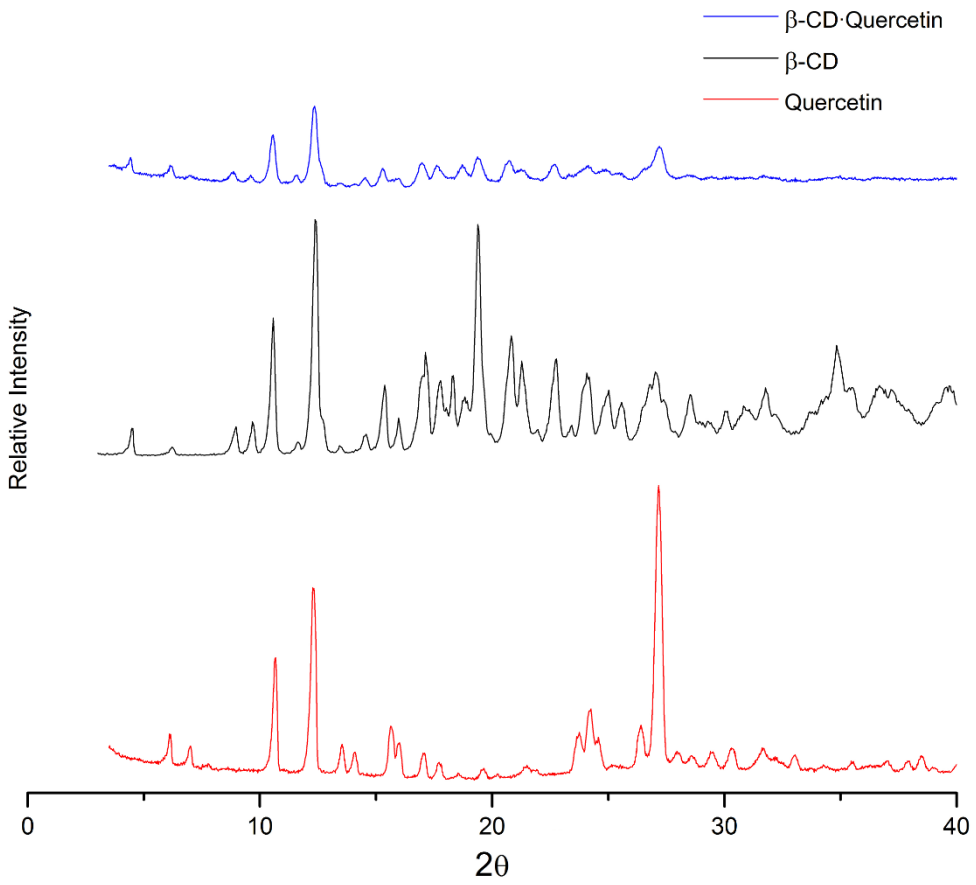


Fig. 13 - PXRD patterns for quercetin, β-cyclodextrin and β-CD·Quercetin.

From the obtained diffractograms, no observable formation of a CD-inclusion compound between quercetin and β-cyclodextrin in the crystalline phase can be perceived. Much due to the reasons stated before, where the obtained product is actually a mixture of free guest and host compounds and CD-inclusion compound, the presence of a crystalline inclusion compound is not visible, indicating that its possible formation is made in an amorphous phase, which can be observed by microscopy (Figure 14).

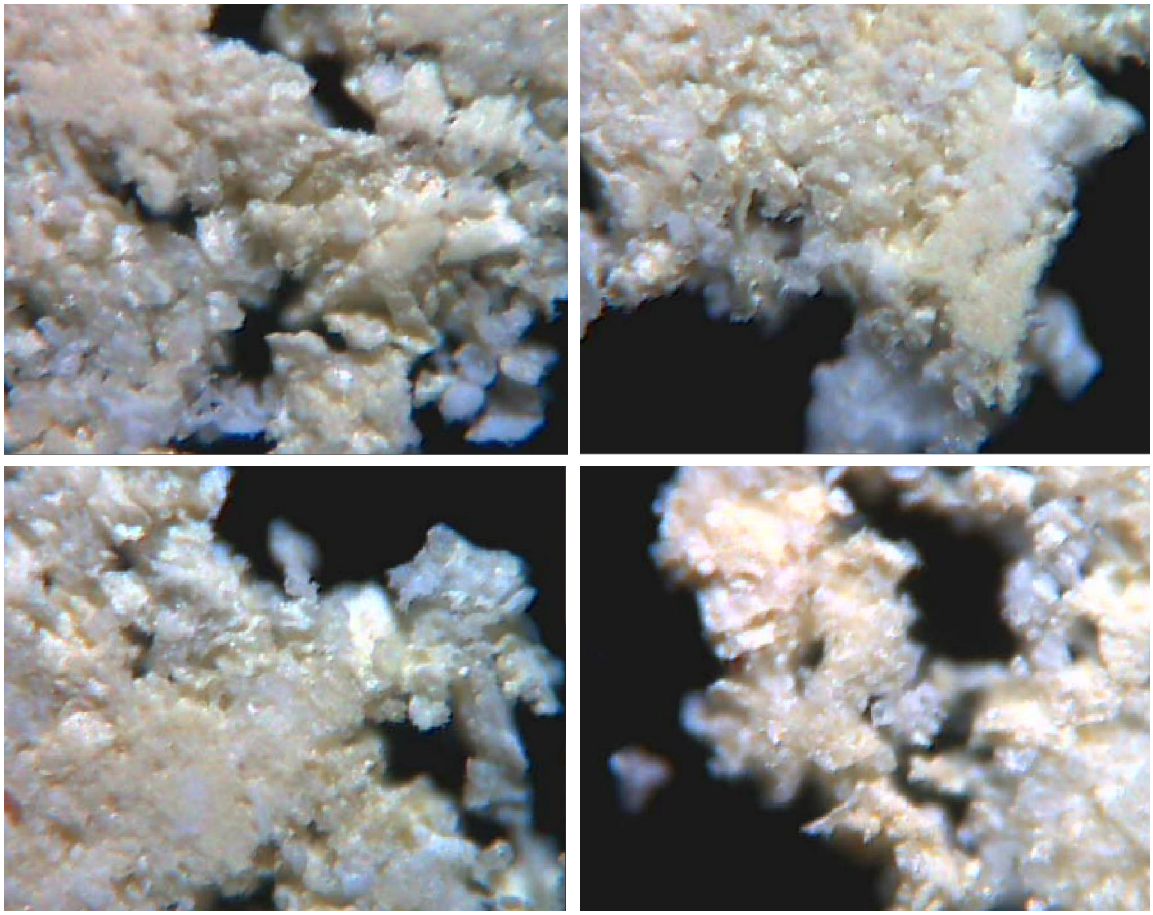


Fig. 14 – Microscopy pictures of the β-CD·Quercetin powder obtained. Magnification: 10x.

γ-CD·Quercetin

The diffractograms for quercetin, γ-cyclodextrin and γ-CD·Quercetin are presented in Figure 15.

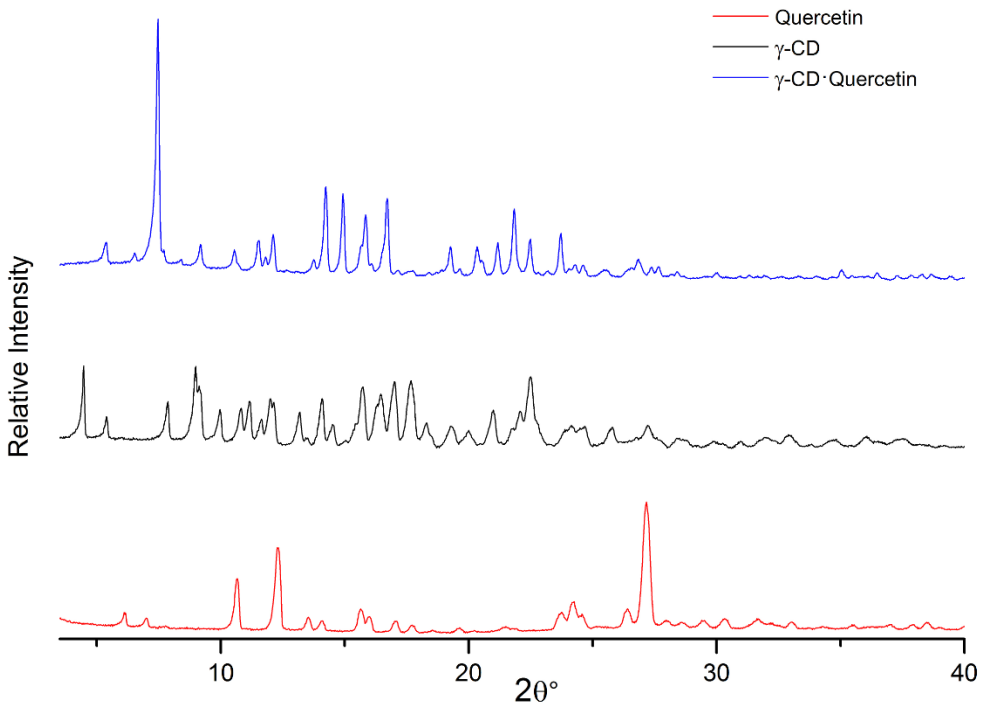


Fig. 15 - PXRD patterns for quercetin, γ-cyclodextrin and γ-CD·Quercetin.

In this case, the formation of a microcrystalline CD-inclusion compound is perceivable from the presence of a trace corresponding to a new phase, without any discernible reflection ascribed to the pure guest and host molecules.

It is also possible to confirm the existence of a CD-inclusion compound through the comparison of the obtained diffractograms with others obtained for known inclusion compounds, calculated from tridimensional coordenates (Figure 16). In this case, is possible to see similarities between the reflexions of the diffractogram obtained for the γ-CD·Quercetin product and the ones from a diffractogram of an inclusion compound of γ-cyclodextrin and crown ether, with an overall similar envelope of reflexion. This indicates that they may share the same tridimensional packing of the host compound, specifically a channel-like conformation.

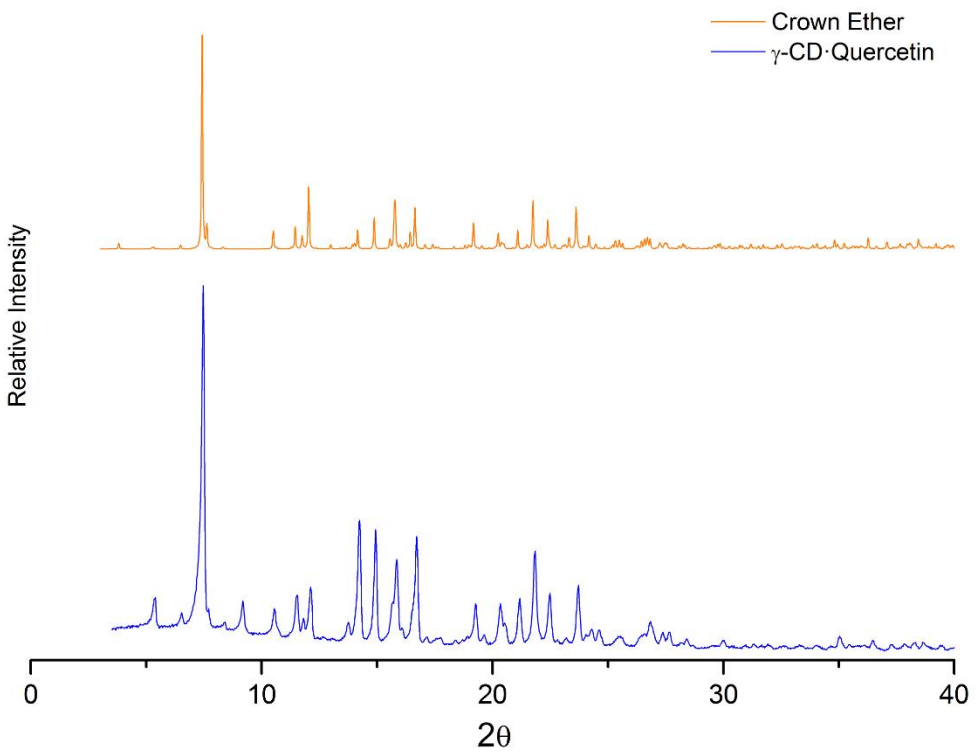


Fig. 16 - Comparison of the PXRD patterns for the γ-CD·Quercetin product and crown ether.

3.2 Antioxidant Assays

3.2.1 TBARS Assay

The TBARS assay is one of the most widely applied methods for determination of lipid peroxidation. Nonetheless it has a few drawbacks that should be taken into account, namely its lack of sensitivity and specificity. Indeed, thiobarbituric acid reacts not only with lipids, but also with sugars, amino acids, bilirubin and albumin, which can interfere in the colorimetric measurement of malonaldehyde (8).In addiction, the heterogeneous nature of egg yolk, which is composed not only by lipids but also by water, proteins, carbohydrates and minerals, is also a source of interferences, further complicating the determination of the lipid peroxidation for a specific substance (9). The TBARS assay is also unable to differentiate between the different types of lipids, and given that egg yolk is composed by a variety of triglycerides, phospholipids, cholesterol and also lipoproteins(9), the determination of the peroxidation of a specific type of lipid is deemed impossible.

In spite of its problems, the TBARS assay remains an easy and inexpensive method for the qualitative evaluation of the peroxidation of compounds. In a first approach, it is quite useful to evaluate if the compound under study affects the reaction detected by thiobarbituric acid, allowing to understand its antioxidant potential before proceeding to more elaborate tests. For the objective of this part of the study, which is to assess if the synthetized complexes are capable of interfering with the oxidation processes that occur in food matrices, this test has proven useful and afforded promising results.

The results obtained for this assay are presented in Figure 17.

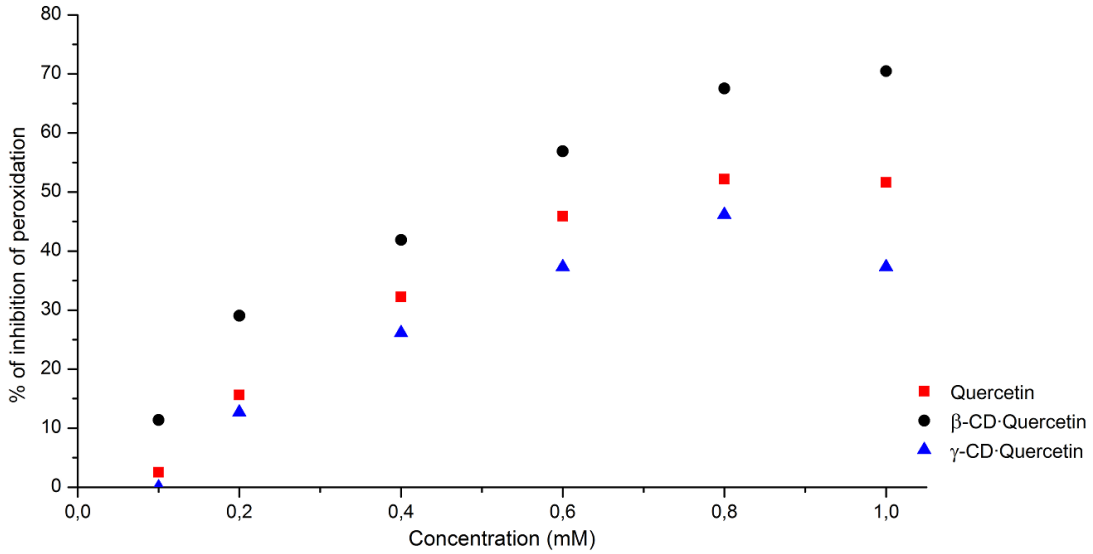


Fig. 17 -% of inhibition of peroxidation by the various compounds under study.

From these results, it is noticeable that all the compounds under study have some anti-peroxidation capacity, with β-CD·Quercetin being the one with the higher percentages of inhibition of peroxidation, followed by quercetin and γ-CD·Quercetin, sequentially. All the compounds increase the inhibition of peroxidation with increasing concentration, albeit at high concentrations, namely from 1 mM and above, the percentage of inhibition lowers slightly, which might be indicative of a pro-oxidant effect occurring at high concentrations of the compounds (well known for these systems). The higher anti-peroxidation activity from the β-CD·Quercetin is expected – considering the results obtained in the characterization of this product, which is composed not only by CD-inclusion compound in an amorphous phase, but also by free quercetin and cyclodextrin, which will have anti-peroxidation activity on their own as well, it is possible that the formation of the CD-inclusion compound grants protection to quercetin, allowing it to have a higher antioxidant activity.

Regarding the γ-CD·Quercetin product, it presents the smallest degree of inhibition of peroxidation. This situation might be due to the great affinity of quercetin by γ-cyclodextrin which, after formation of the CD-inclusion compound, may interfere with the antioxidant capacity of quercetin. The occurrence of a “sequestrating” effect of quercetin

by the cyclodextrin is also possible, preventing the flavonoid from having noticeable antioxidant activity in the period of time comprised in this assay.

The results of this assay show that the CD-inclusion compounds investigated are promising new food ingredients which can be used in preventing peroxidation processes, with β-CD·Quercetin having the most interesting performance.

3.2.2 DPPH Assay

The results for the DPPH assay are presented in Figure 18.

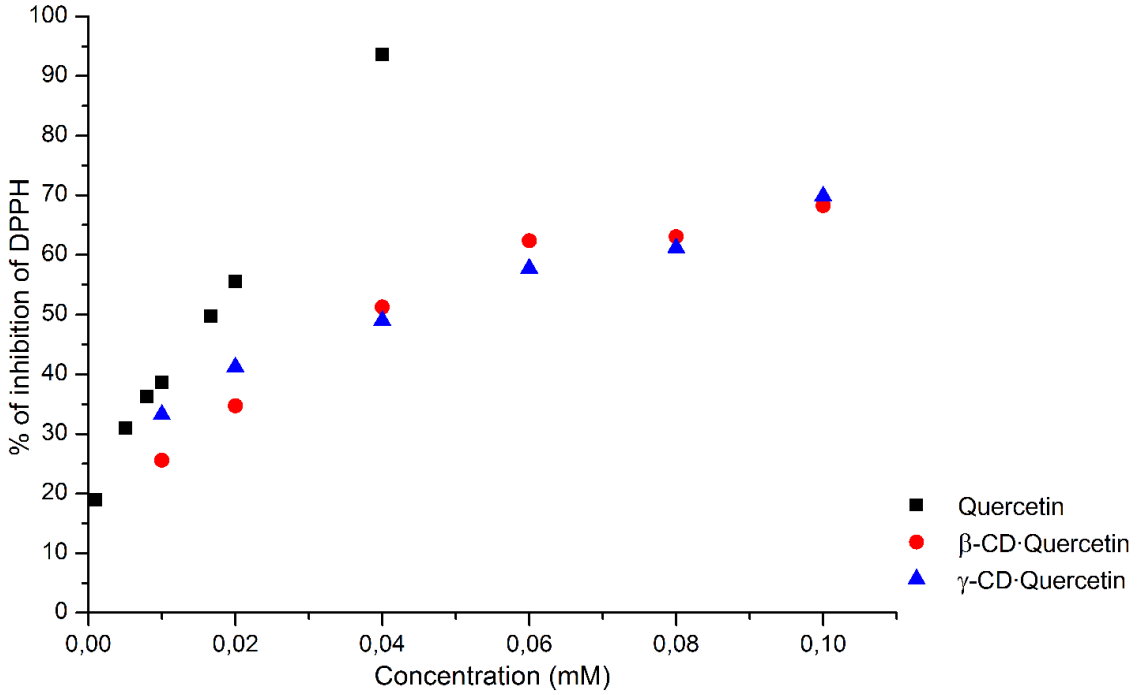


Fig. 18 -% of inhibition of DPPH radicals by the various compounds under study.

From the results of the inhibition of DPPH radicals, it is possible to calculate the IC_{50} for each compound (concentration necessary to reduce 50% of the DPPH radicals). After determination of this value, the procedure is repeated for this specific concentration, in

order to find the t_{IC50} value (time necessary to reach the IC_{50}). From these results, it is then possible to calculate the antiradical efficiency of the compound (Table 14).

Table 14- DPPH parameters and antiradical efficiency (AE) of the various compounds in study.

Compound	***IC_{50} (mM)***	***t_{IC50} (min)***	***AE***
Quercetin	0.017	54	1.09
β-CD:Quercetin	0.05	14	1.43
γ-CD:Quercetin	0.05	16	1.25

The CD-inclusion compounds studied present very similar curve profiles, which indicates that their kinetics of reaction are quite similar. From Figure 17 it is also possible to understand that quercetin acts as an radical scavenger in a very efficient way at the lowest concentrations, while the CD-inclusion compounds require higher concentrations to display antioxidant activity and scavenge the radicals in a more steady and gradual manner. The compound with the higher antiradical efficiency is β-CD·Quercetin, followed by γ-CD·Quercetin and quercetin, orderly. These results are due to the fact that quercetin has a lower IC_{50} (*i.e.* scavenging the same amount of radicals in lower concentrations). For concentrations above 0.04 mM, quercetin reaches a plateau, and it will eventually begin to behave as a pro-oxidant over time. Regarding the CD-inclusion compounds, even though these demand higher concentrations in order to have acceptable antioxidant activity (which is understandable given that the main antioxidant component is not completely exposed to the radicals, and also because the molecule in question is, by itself, heavier), they have very good results of antiradical efficiency, having a faster kinetic of reaction and reaching the IC_{50} in less than half of the time needed for quercetin, which can arise from some antioxidant activity from the host molecules, with the cyclodextrin possibly including some radicals in its cavity.

Regarding the antiradical efficiency (AE) values, there are currently no available reference standard tables which allow correlating the AE with a high or low antioxidant capacity. This leaves the interpretation of the results to comparisons between the compounds herein under study and compounds of well-known antioxidant capacity. There are some remarks, however, to be made about the DPPH assay. Even though it is one of the most widely applied methods for determining antioxidant capacity, there are some

details that would benefit from improvement. Currently, there is no standardization of this assay, meaning that there is a wide range of protocols published, each one differing in solvent, DPPH concentration and sample concentration range. There are no stated mechanized protocols that refer to the fair concentrations of DPPH to be used, or the most suitable solvent, or the recommended DPPH/antioxidant ratio. A.L. Dawidowicz *et al*. have analysed the various practical problems in the estimation of antioxidant activity of compounds by the DPPH· method, finding various parameters that are prone to influence the results (10): according to them, the antiradical efficiency of a compound, estimated by the DPPH method, depends on the type of solvent used for the dissolution of the antioxidant compound, increases with the water content, decreases with metal ion concentrations and depends on the pH (with higher pH achieving faster rates of the reaction between DPPH and the antioxidant). This hints at the possibility of modelling the results towards desirable outcomes, through the manipulation of the various factors that influence antiradical efficiency. A higher antiradical efficiency can be obtained using lower DPPH concentrations, or aqueous solvents with higher pH. From this, extrapolating the actual antioxidant capacity of a compound through this method becomes a complex task of balancing the variables that influence the results, and even though calibrations and optimizations are made to the reagents before the assays, without a verified and unified source material to regulate the protocol used the results obtained can be made to meet the desirable expectations (which is clearly a drawback).

The concept of *antiradical efficiency* parameter, first introduced by Sánchez-Moreno *et al* (11), also warrants standardization. Currently, there is no standard table to correlate the degree of antioxidant capacity with the antiradical efficiency, i.e., there are no references to help understand whether an antiradical efficiency of 1 implies high or low antioxidant capacity. Indeed, these values vary according to the concentration units used. In the table reported by Sánchez-Moreno *et al*, for instance, the antiradical efficiency is expressed in g of antioxidant *per* kg of DPPH, a very uncommon unit.

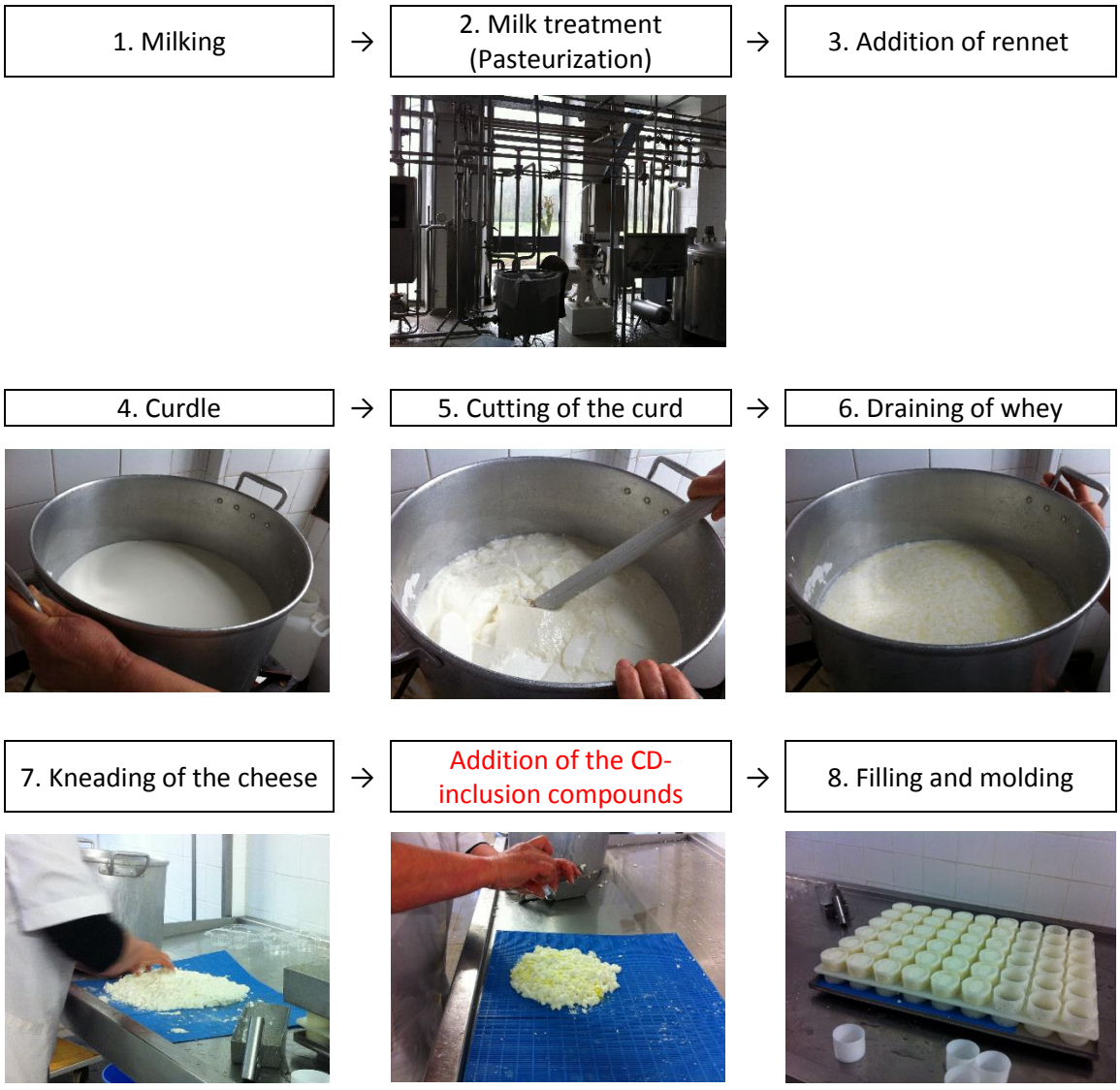
Despite all of the above, the DPPH assay remains a good method to determine the antioxidant capacity of a compound, for it is based on a well-known radical mechanism, and it provides information about the order of the kinetics and the rate of the reaction

involved. It is a useful and practical tool, standing out as one of the most reported and reliable amidst the vast myriad of available methods for antioxidant activity evaluation. Nevertheless, the method will benefit (as stated above) from further improvements, namely the systematization of its protocols (units and reactants) and of the classification of the obtained results.

3.3 Incorporation of the Prepared Compounds in Dairy Products

3.3.1 Manufacture of the Cheeses

In addition to the physical-chemical characterization and antioxidant activity evaluation of the prepared compounds, an incorporation into a food matrix was carried out. The chosen food was fresh unripen cheese, which is produced regularly at the Technological Diary Plant of Coimbra College of Agriculture. Scheme 7 shows the process of manufacture of this product.



Scheme 7 - Flowchart of the production of the fresh cheese.

The milk is first obtained by milking, being pasteurized afterwards to secure microbiological safety. In order to promote the formation of the curd, CaCl (0.6mL/L), powdered milk, NaCl (0.9%) and animal rennet (90% chymosin and pepsin) are added to the milk, and it is let to rest at 32/33°C for 15-20min for curdle. After cutting the curd, which will help the release of the whey, the cheese rests for 5 min, and is then kneaded to further remove whey and other liquids. The inclusion compounds are added after this step, by simply sprinkling the powders into the unmolded cheese. The resulting product is then put in molds and into the fridge for some hours until ready for consumption. β-CD·Quercetin was added in a 0.029% ($^{m}/_{m}$) concentration and γ-CD·Quercetin in a 0.043% ($^{m}/_{m}$) concentration, values that are completely safe for human consumption according with the GRAS notices for these compounds (references 140 and 141 from introduction).

3.3.2. Organoleptic Characteristics of the Treated Cheeses

The addition of the CD-inclusion compounds, even though in such low percentage, produced some changes in the organoleptic characteristics of the cheeses, in comparison with the untreated cheeses from the same batch. In general, the most noticeable changes following the addition of the compounds were noted in the texture. Cheeses which contained the CD-inclusion compounds were more compact and less “watery”, with a more cohesive structure. This can be easily explained - considering that cyclodextrins are able to retain water molecules, it is likely that when they come in contact with the water molecules in the milk they will avoid its release, thus resulting in a dryer surface. In appearance, the treated cheeses presented small yellow dots, which resulted from the heterogeneous dissolution of the coloured CD-inclusion compound in the cheese mixture. This was more prominent in the cheese with the γ-CD·Quercetin product, and a possible explanation is the fact that γ-CD is less hydrophobic that β-CD, and given that the cheese is a mainly apolar medium, the dissolution of γ-CD·Quercetin product was less efficient. In terms of odour, there were no significant differences between the treated and untreated cheeses, and regarding taste, the treated cheeses presented a more intense bitter flavour, while the untreated ones were considered mild and plain.

3.3.3 Sensory Analysis of the Cheeses

As before mentioned, a sensory analysis was conducted to understand if the addition of the prepared CD-inclusion compounds produced significant changes to the cheeses. Regarding the fresh cheese treated with the β-CD·Quercetin product, the overall opinion stated that the flavour of the treated cheese was more intense, having a more bitter taste and a stronger tang. About the cheese to which the γ-CD·Quercetin product was added, the comments were similar to the previous, with many mentions about the stronger and more bitter flavour of the treated cheese. The preference between the not treated and the treated cheese varied a lot with the personal preference of the taster, and given that this is a non-trained panel and a big number of tasters is necessary to evaluate if the addition of the CD-inclusion compounds altered significantly the food product, tasters with preference for stronger and more intense cheeses would go for the treated variant, while those who preferred more subtle and soft cheeses chose the not treated one.

The Pearson's chi-squared test for independence was applied to the results, for this test allows to determine whether there is a significant difference between two or more variables. The results are shown in Table 15.

Table 15 - Results from the preference test.

Type of compound	*Not treated*	*Added compound*	*No preference*	*Total observations*[1]
β-CD·Quercetin	14	15	1	29
γ-CD·Quercetin	10	15	5	25

[1]The "no preference" observations were not considered for the total number of observations, for they are not relevant to access whether the significant difference between the products.

Considering 1 degree of freedom, the p-value calculated for the β-CD·Quercetin cheese was 0.7, while for γ-CD·Quercetin it was 0.4. Giving that these values are higher than 0.05, it is considered that the variables in study are not significantly different, which means that previously described to the organoleptic characteristics of the cheeses which were identified upon the addition of the compounds, do not have significant statistic relevance. This outcome can be viewed in two perspectives – if the objective was to create a new and organoleptic improved product, the addition of the compounds did not manage to achieve such task, but if the objective was to improve the nutraceutical and technological

characteristics of the product, such as the expiry date, without altering significantly the product, then the addition of the compounds came out with positive results.

3.3.4 Evaluation of Potential Use as Preservative

In order to evaluate the effect of the addition of the CD-inclusion compounds in the preservation of the cheeses, the pH of the cheeses was measured periodically for a time of 21 days, and the results are presented in Table 16.

Table 16 – Progression of the pH values of the various cheeses, from 0 to 21 days of storage at 4°C.

	0	***7***	***14***	***21***	***Mean***
Not treated	6.48	5.50	4.79	4.75	5.38
β-CD·Quercetin	6.62	5.73	4.85	4.56	5.44
γ-CD·Quercetin	6.64	5.79	4.77	4.62	5.45

The results presented in the table show that, over time, there is a slight decrease in the pH of the cheeses. This may be due to fermentation of residual lactose to lactic acid by heat stable indigenous bacteria in milk, which survive pasteurization and cheese production or are present by post-manufacturing contamination. It may also be associated with the very limited production of alkaline products related to low protein-breakdown (12). In the first 2 weeks, the pH of the cheeses with the incorporated complexes is slightly higher than the pH of the untreated cheeses, which may imply that these, in an early stage, manage to better preserve the stability of the compounds and have better quality than the not treated ones. Overall, the state of conservation of both types of cheeses was very similar, which means that the CD-inclusion compounds added had no significant effect in the preservation of the products.

References

1. Cornard JP, Merlin JC, Boudet AC, Vrielynck L. Structural Study of Quercetin by Vibrational and Electronic Spectroscopies Combined with Semiempirical Calculations. Biospectroscopy. 1996; 3(3):183–93.

2. Borghetti GS, Lula IS, Sinisterra RD, Bassani VL. Quercetin/beta-cyclodextrin solid complexes prepared in aqueous solution followed by spray-drying or by physical mixture. AAPS PharmSciTech. 2009; 10(1):235–42.

3. Giordano F, Novak C, Moyano JR. Thermal analysis of cyclodextrins and their inclusion compounds. Thermochim Acta. 2001; 380(2):123–51.

4. Da Costa EM, Filho JMB, do Nascimento TG, Macêdo RO. Thermal characterization of the quercetin and rutin flavonoids. Thermochim Acta. 2002; 392-393:79–84.

5. Ribeiro A, Figueiras A, Santos D, Veiga F. Preparation and solid-state characterization of inclusion complexes formed between miconazole and methyl-beta-cyclodextrin. AAPS PharmSciTech. 2008; 9(4):1102–9.

6. Caira MR. On the isostructurality of cyclodextrin inclusion complexes and its practical utility. Revue Roumaine de Chimie. 2001; 46(4): 371-86.

7. Naidu NB, Chowdary KPR, Murthy KVR, Satyanarayana V, Hayman a R, Becket G. Physicochemical characterization and dissolution properties of meloxicam-cyclodextrin binary systems. J Pharm Biomed Anal. 2004; 35(1):75–86.

8. Knight JA, Robert K, Mcclellan L. Specificity of the Thiobarbituric Acid Reaction: Its Use in Studies of Lipid Peroxidation. Clin Chem. 1988; 34(12):2433–8.

9. Anton M, Composition and Structure of Hen Egg Yolk. In: Huopalahti R, López-Fandiño R, Anton M, Schade R, editors. Bioactive Egg Compounds. Heidelberg: Springer-Verlag Berlin Heidelberg.

10. Dawidowicz AL, Wianowska D, Olszowy M. On practical problems in estimation of antioxidant activity of compounds by DPPH method (Problems in estimation of antioxidant activity). Food Chem. 2012; 131(3):1037–43.

11. Larrauri A, Saura-calixto F. A Procedure to Measure the Antiradical Efficiency of Polyphenols. J Sci Food Agric. 1998; 76:270–6.

12. Torres N, Chandan RC. Flavor and Texture Development in Latin American White Cheese. J Dairy Sci. 1981; 64(11):2161–9.

4. Conclusion

The present work describes the inclusion of a flavonol, quercetin, into the cavity of two cyclodextrins, β and γ. The resulting products were characterized regarding the geometry of interaction, at the molecular level, by means of a wide variety of techniques: vibrational spectroscopy (FTIR and Raman), thermogravimetric analysis and powder x-ray diffraction. The effect of the inclusion process on the antioxidant potential of quercetin was evaluated, by several standard assays. Finally, a possible application of these products in food was studied by incorporating them into a portuguese fresh cheese and determining their effect in the organoleptic characteristics of this food product.

Through this study it was possible, as mentioned earlier, to investigate the relative geometry inclusion of quercetin inside the cyclodextrins cavity, being found that quercetin is included in great depth into the cavity of γ-cyclodextrin. For the smaller β-cyclodextrin, it was not possible to obtain a pure inclusion compound, with an amorphous mixture being obtained instead. It was also observed that γ-cyclodextrin is able to exert thermic protection of the guest molecule.

The antioxidant tests performed revealed that the CD-inclusion compounds have an antioxidant activity slightly different from that of the pure guest molecule, quercetin. The TBARS assay showed that these have anti-peroxidation activity, with β-CD·Quercetin demonstrating the highest percentage of peroxidation inhibition, and the DPPH assay showed that the radical scavenging capacity of the CD-inclusion compounds occurs faster, but demands higher concentrations in order to have a reasonable antioxidant activity.

Incorporation of the CD-inclusion compounds into fresh cheese demonstrated that these are able to alter some of its organoleptic characteristics, even in low concentrations. Considering the sensory analysis performed, the product was not rejected by the tasters, and was overall accepted, which might indicate that this food product may have some commercial application. Their potential use as preservatives, however, did not show to be very promising, as the pH and texture of the product did not change overtime between treated and untreated cheeses.

4.1 Future Work

There are some possible areas of future research regarding the concepts addressed in this work. Concerning the characterization of the inclusion compounds, it would be interesting to optimize the crystallization method, in order to obtain crystals suitable for Single-crystal X-ray Diffraction, which would retrieve accurate information about the unit cell, bond-lengths, bond-angles and site-ordering of the inclusion compounds. There is also room for improvement regarding the preparation of a pure β-CD·Quercetin inclusion compound, for this has revealed to be a very dynamic and complicated system that would need further adjustments and research. Actually, this compound yelded the best results in the anti-peroxidation assay, presenting promising potential as a food preservative. Regarding the antioxidant potential, a more in-depth antioxidant capacity analysis can be achieved, through the application of more antioxidant assays that approach different antioxidant reactions and mechanisms.

As to incorporation of this type of compounds in food products, it would certainly be useful to attempt higher concentrations, testing in different products and analysing the potential as preservatives through the use of, for example, Preservative Efficacy Testing assays.